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SOME EFFECTS OF TEMPERATURE ON THE GROWTH OF *CHARA ZEYLANICA* WILLD.

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Members of the Characeae have been used extensively in various investigations including ion accumulation, cyclosis, and cytoplasmic permeability, yet investigations on mineral nutrition and the effects of light and temperature have been meager. Concerning temperature effects Karling, 1924, using cultures of *Chara fragilis* Desvaux, concluded that formation of reproductive structures depended primarily on the length of day, and secondarily on temperature. The purpose of the present investigation was to determine certain growth responses of another species of *Chara* to the influence of temperature.

Fritsch, 1948, may be consulted for an account of the morphology of *Chara*. However, the nomenclature used in this paper for discussion of reproductive organs was used originally by Lindley, 1830, and adopted currently by Smith, 1955.

MATERIALS AND METHODS

In September 1954, specimens of *Chara* were collected from three areas in Nebraska: (1) a small pool near Arabia; (2) the Gretna Fish Hatchery; and (3) the Platteview Recreation Grounds in Louisville. The plants were identified by Mrs. Fay Kenoyer Daily of Butler University, Indianapolis, Indiana, as *Chara contraria* A.Br. (1 and 2 above), and *Chara zeylanica* Willd. (3 above). Herbarium specimens are filed at Butler University and at the University of Nebraska. The plants were established in aquaria containing tap water and approximately one inch of common garden soil covered with a half-inch surface layer of white quartz sand. Aquaria were located in a north window, and additional continuous light was furnished by a fluorescent lamp. One collection of *C. contraria* grew rapidly under these conditions but soon died. The other collection of *C. contraria* like *C. zeylanica*, showed a moderate rate of growth. Preliminary attempts to grow excised portions anchored only in quartz sand showed that *C. zeylancia* grew equally well in tap water or in nutrient solution, while the excised portions of *C. contraria* grew slowly or not at all. For this reason *C. zeylancia* was chosen as the experimental plant for this study.

One plant of *C. zeylanica* was selected from an aquarium and planted in a large battery jar containing soil covered by sand as mentioned above. It was kept in a windowless basement room in which the temperature was fairly uniform. An incandescent tungsten lamp burning 16 hours a day provided the only illumination and kept the temperature of the culture at about 27°C. As the plant developed, the lateral branches of unlimited growth were removed and replanted in the same battery jar where they were allowed to grow until the substrate was covered by many shoots. Thus the experimental plants comprised a single clone. After these shoots produced a luxuriant growth, branches were severed at internodes and planted in the experimental set-up with the lowest node buried in quartz sand. Measurements of growth included neither the buried node nor the internode above it. When the test shoots were planted the number of nodes and plant length were recorded.

Constant temperature tanks, located in a greenhouse, were maintained at 16°, 20°, 24°, 28°, and 32°C. These temperatures were maintained in a series of five tanks, each containing four stainless steel cans designated "A," "B," "C," and "D." Approximately one inch of white quartz sand was placed in each can which was then filled with tap water. Two clonal members of *C. zeylanica* (each bearing five nodes) were planted in can "A" in each of the five different temperature tanks. One clonal member was planted in each of the other three cans "B," "C," and "D" of each of the five tanks. All plants received daylight supplemented by a 100-watt bulb placed over each tank. A time clock regulated the light period at 16 hours per day The experiment began February 13, 1955, and the final observations were made April 1 of the same year.

RESULTS

Increments of growth are shown in Table I. Many of the main shoots ceased to grow during the course of the experiment and became covered with a layer of filamentous green and blue-green algae. A brown scum was also present on many plants. Only one plant died before the first growth increment was measured. On many plants held at 20° and 32°C. and on all plants at 28°C. the main shoots had stopped growing before the conclusion of the experiment. Of the 12 plants in which this occurred, seven plants (3 at 32°, 3 at 28°, and 1 at 24°C.) developed lateral branches whose lengths per plant totaled more than the growth of the main shoot. One of the seven showed the greatest total growth increment (46.9 cm.) with development of 37.2 cm. laterals, while the main shoot increased its length only 9.7 cm. The five remaining plants (2 at 28°, and 3 at 20°C.) developed laterals totaling less than the length of the main shoot before the latter ceased growing. The production of laterals also accounted for the largest sum of growth increments (154.4 cm.) for all five plants in any one temperature bath at 32°C. How-

TABLE 1

Growth increments of *Chara zeylanica* at various constant temperatures.

Temp. C.	Plant	Main shoot cm.	Lateral branches cm.	Total growth cm.	No. nodes main shoot	No. nodes lateral branches	Total No. nodes	Rhizoidal mass main shoot cm.
16	A_1	0.2	0.0	0.2	0	0	0	1.0
	A_2	0.0	0.0	0.0	0	0	0	0.9
	B	0.2	0.0	0.2	0	0	0	0.3
	C	0.1	0.0	0.1	0	0	0	0.5
	D	0.1	0.0	0.1	0	0	0	0.3
20	A_1	6.5*	0.0	6.5	4	0	4	4.0
	A_2	5.2*	5.1	10.3	3	5	8	2.0
	B	15.0	1.3	16.3	8	2	10	5.0
	C	9.3*	0.0	9.3	4	0	4	3.8
	D	— **	—	—	—	—	—	—
24	A_1	23.4	14.7	38.1	11	10	21	6.0
	A_2	12.7*	20.2	32.9	6	15	21	8.0
	B	21.3	15.6	36.9	12	14	26	7.0
	C	16.7	3.5	20.2	10	3	13	12.0
	D	15.3	5.5	20.8	9	4	13	8.0
28	A_1	9.7*	37.2	46.9	5	21	26	4.0
	A_2	5.8*	12.5	18.3	4	11	15	2.5
	B	5.1*	0.0	5.1	3	0	3	1.5
	C	6.6*	1.0	7.6	4	3	7	3.5
	D	7.8*	20.5	28.3	5	15	20	3.0
32	A_1	5.2*	25.5	30.7	3	19	22	4.0
	A_2	4.0*	15.5	19.5	4	21	25	3.2
	B	24.0	19.5	43.5	12	15	27	3.0
	C	6.8*	18.8	24.2	3	11	14	2.0
	D	26.0	10.5	36.5	13	12	25	3.0

* indicates those plants, the main shoots of which ceased development during the course of the experiment.
** indicates plant that died soon after planting.

ever, many of the lateral branches which developed on these plants consisted of small outgrowths that appeared to be stunted and did not develop "leaves." In all of the plants whose main shoots were actively growing at the end of the experiment, the growth of the main shoot exceeded the growth of its laterals. The greatest total growth of such a plant (43.5 cm.) occurred at 32°C. with the main shoot contributing 24.0 cm. and the laterals 19.5 cm. The largest increment of growth of the main shoot (26.0 cm.) also occurred at 32°C. The most consistent growth of main shoots, however, occurred at 24°C., at which temperature the increments of main-shoot growth for all 5 plants totaled 89.4 cm. in contrast with 66.0 cm. at 32°, 36.0 cm. at 20°, and 35.0 cm. at 28°C. This consistent growth of the main shoot at 24°C., combined with a sum of 59.5 cm. growth of laterals, accounted for the second largest sum of growth increments (148.9 cm.) for all five plants in one temperature bath. The only significant growth increment which increased with temperature was that of the sum of the laterals at one temperature. Laterals totaled 6.4 cm. at 20°, 59.5 cm. at 24°, 71.2 cm. at 28°, and 89.8 cm at 32°C.

A record was kept of the number of nodes produced by each plant in order to check the growth increments for evidence of excessive elongation. The total number of nodes developed by all five plants in a temperature bath were as follows: 113 at 32°, 94 at 24°, 71 at 28°, and 26 at 20°C. The total number of nodes which developed on laterals increased with a rise in the temperature of water baths: there were 7 nodes at 20°, 46 at 24°, 50 at 28°, and 78 at 32°C. This corresponds to the increased growth of laterals at the same temperatures. A similar relationship existed between the growth of main shoots and the number of nodes produced on the main shoots of all plants grown at a given temperature. Total new nodes on the main shoots were 48 at 24°, 35 at 32°, 21 at 28°, and 19 at 20°C. Although there were a few more nodes developed on the plants at 32°C. than at 24°C., a greater number of nodes developed on the main shoots at 24°C.

Development of the rhizoidal system was greatest at 24°C.; the plants, producing a heavy entangled mass, attained a total length of 41.0 cm. for the five plants. The shortest rhizoidal system on any plant at 24°C. (6 cm.) was longer than the maximum length attained at any other temperature. Total lengths for the remainder of the plants at the other temperatures were 15.2 cm. at 32°, 14.8 cm. at 20°, 14.5 cm. at 28°, and 3.0 cm. at 16°C. Rhizoidal development at 16°C., however, consisted of only one or two short filaments per plant.

Secondary protonemata which developed on the experimental plants had the form of lateral branches. They originated from the nodal region, but were soon distinguished as protonemata by their smaller "leaves," longer internodes, and their downward growth to the substratum. Measurements of secondary protonemata were included under lateral branch measurements in Table I. At 20°C. the plants produced secondary protonemata to the exclu-

sion of lateral branches. At higher temperatures secondary protonemata as well as lateral branches were formed by the main shoot. At 28°C. all plants developed lateral branches which, in turn, produced secondary lateral branches.

At 16°C. the growth of shoots and the production of nodes and secondary protonemata was either absent or negligible, and was therefore not mentioned in the above paragraphs.

Of the five plants in each temperature bath, three plants each at 16°C., three at 32°C., one plant at 24°C., and one plant at 28°C. produced reproductive structures, but none were produced on plants in the 20°C. bath. These structures were borne on new growth of the main shoot of plants subjected to 24°C. and 32°C., as well as on branches of unlimited growth at 28°C. In the 16°C. bath, however, the plants failed to grow appreciably in length, and reproductive structures were produced on the main shoot of the original material.

Nucules developed beyond the yellow, linear stage, becoming spheroidal and white, only on plants grown at 32°C. At this temperature, however, fertilization apparently did not occur since the oogonial wall did not turn black (3).

DISCUSSION

The temperatures chosen were those which were easily maintained and which gave promise of vegetative growth. The low temperature of 16°C. was maintained by a flow of tap water through the tank; facilities for refrigeration were not available for maintaining lower temperatures. Copeland, 1936, reported the maximum temperature record for Characeae growing in hot springs of Yellowstone National Park to be 38.1°C. He noted that Prat, 1929, also working in Yellowstone, recorded epiphytes on *Chara* growing in tepid water. Karling, 1924, stated that cultures of *C. fragilis* soon deteriorated and died at constant temperatures above 32°C. which therefore was chosen as the maximum temperature in this study.

Although, as previously stated, the greatest total growth occurred at 32°C., the growth rate was irregular. The main shoots developed rapidly at the beginning of the experiment but ceased to grow after lateral branches were initiated. This may have been due in part to the relatively low nutritional status of the experimental plants which grew in tap water and were anchored in quartz sand. Algal contaminations of the experimental plants were also most severe at 32°C. The possibility that changes of other environmental conditions might permit maintenance of apical growth of the main shoot, development of a larger rhizoidal system, and still make use of the warmer temperature that stimulates lateral branch development was not investigated in this study. Development of the main shoot for all five plants at one tem-

perature was most rapid and constant at 24°C. This tended to emphasize that main shoot growth at this level of nutrition progressed more rapidly at a lower temperature than that which gave maximum lateral branch development under the same nutritional conditions. However, high and low temperatures may not be the only factors which adversely affect the continuous growth of the apical cell of the main shoot. Even in *Chara*, a thallophyte, the activities of the apex may have an inhibitory effect on the development of laterals, since in all plants irrespective of temperature, in which the apical cell was growing at the end of the experiment, the growth of the main shoot exceeded the total growth of its laterals. The most striking feature shown by the data was increased lateral branch development with increase of temperature. Lateral branches and secondary protonemata were not developed in direct response to the growth of the main shoot as was clearly demonstrated by the lack of correlation between the number of nodes developed on the main shoot compared with those developed on laterals in any one temperature bath.

Ratios of growth in length to number of nodes produced for each plant varied widely for plants grown at 20°, 28°, and 32°C., but were more closely grouped at 24°C. This indicated a more consistent growth in length per number of nodes produced at 24°C. than at other temperatures. These ratios when determined for laterals and main shoots separately for each plant at each temperature showed that in general the laterals produced less growth per node while the main shoots produced more growth per node developed. This helped to establish that growth increments as measured for a small group of plants in this experiment were nearly as valid as an index of plant growth as was the number of nodes produced. Ordinarily the number of nodes produced would be considered more significant since they are produced with remarkable regularity by the activity of the apical cell which in *Chara* produced a cell that divides to form a node and an internode.

The rhizoidal development which occurred at 24°C. was nearly three times greater than the moderate growth at 20°, 28°, and 32°C. Importance of the rhizoidal system has been shown by Vouk and Benzinger, 1929, who concluded that the rhizoids were the main organs of absorption of nutritive materials and that the thallus surface had only a subordinate function in this respect. Rhizoids are, perhaps, a better index of vegetative growth than are shoots, for they are not directly affected by visible algal contaminations as are the shoots.

Under the conditions maintained in this experiment, 24°C. gave the greatest growth for the main shoots and rhizoids and the most consistent over-all growth of the main shoots and lateral branches.

The production of reproductive structures in only a few plants minimizes their value as indicators of growth rate in this experiment. Transeau, 1903, stated that in certain algae a vegetative period is necessary before reproduc-

tion occurs, and the approach of the reproductive period in *Spirogyra* sp. is accelerated or retarded by temperature, light intensity, and concentration of the mineral content of water. Although plants in the 16°C. bath showed little vegetative growth, indicating a retarding effect of temperature, reproductive structures were not prevented from developing. Karling, 1924, found that reproductive structures were borne on *C. fragilis at* 2°C. under constant illumination. He also found that nucules aborted at 32°C. This agrees with the findings of the present study. Since reproductive structures were formed during the experiment at most of the temperatures used, it would seem, as stated earlier by Karling, 1924, for *C. fragilis,* that temperature is not the primary factor in formation of reproductive structures.

SUMMARY

1. Clonal members of *Chara zeylanica* were grown at five different constant temperatures (16°, 20°, 24°, 28°, and 32°C.). Growth of the main shoots and lateral branches were recorded separately for increase in length and the number of nodes produced. The length of the rhizoidal mass was recorded for each plant at the end of the experiment and notations were made of the presence of reproductive structures.

2. Under the conditions of the experiment the most abundant growth of main shoots and of the rhizoidal system occurred at 24°C.

3. Growth at 16°C. was restricted, while at higher temperatures main shoots ceased to grow. Production of lateral branches increased with an increase of temperature but at 32°C. was limited, for the most part, to small outgrowths. Growth increments were nearly as valid as the number of nodes produced as an index of plant growth.

4. Reproductive structures developed on plants growing at all temperatures except 20°C.

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VARIATIONS IN ENGINEERING FEATURES OF THE NESTS OF SEVERAL SPECIES OF BIRDS IN RELATION TO NEST SITES AND NESTING MATERIALS

by

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During about thirty-five years of field work in bird ecology in eastern and central North America, I have examined in the field or collected and analyzed about 20,000 nests of 169 species of birds. This list represents nearly a third (31.3% of the 533 species listed by Pough for the entire region of eastern and central North America from southern Texas to central Greenland. All of my studies have been confined within the region between the latitude of 30° and 50° north and between the 100th meridian and the Atlantic Ocean. The largest amount of my field work and study has been done in five counties of southeastern Michigan and three counties of southwestern Ontario where 25,379 nesting records have been established for 143 species of birds by about fifty field observers of the Detroit Audubon Bird Survey in the last 12 years.

In the course of these studies, I have been impressed repeatedly by the ability of several species of birds to construct successful nests on sites which from a structural standpoint have exhibited considerable variability. When sites, nests and nesting materials are carefully examined, the facts suggest that most birds are unable to vary to any great degree from instinctive engineering activities and patterns of building. A breakdown of the nest types and sites of the 533 species listed by Pough, 1946, 1951, shows that 496 species (93.5%) build statant or standing nests either in trees, other vegetation, artificial sites, or on the ground. Of these statant nest-builders, about 51% construct nests on or in the ground, or lay their eggs on the ground without gathering nest materials. The remaining 49% of the statant nest-builders construct or use nests in trees, shrubs, or forbs. Of these species nesting above ground in vegetation about 60 species build nests that are attached to their sites by grasses, bark, lichens, spider webs, and other fibrous materials. About a third of the attached nests are usually pensile or pendulous. Twelve

species of birds nest on floating mats of vegetation and three species build nests which are adherent to the perpendicular surfaces of cliffs, walls or chimneys.

The study of a large number of nests of all types in their original sites, and in numerous habitats, has revealed that significant engineering variations appear chiefly in those statant nests in vegetation above the ground, and to a lesser extent, those which are adherent. Another variation in statant nests is called saddling. About 40 species, or roughly 7% of the bird species of eastern North America, exhibit considerable variation in the engineering features of their nests.

Three important factors appear to be involved in the nest placement of species of birds which demonstrate engineering variations: 1. The type of nest, 2. the type of nesting materials used, and 3. the type of site on which the nest is built. The nighthawks and whippoorwills make no attempt to build elaborate nests but simply lay their eggs on the ground or on leaves of the forest floor. Some terns and shorebirds wallow out saucer-shaped depressions in the earth. These depressions may or may not be lined. The Prairie Horned Lark (*Otocoris alpestris*) and the Vesper Sparrow (*Pooecetes gramineus*) excavate cup-like depressions in the ground in which they construct nests as complex as those of many tree-nesting birds like the Mourning Dove (*Zenaidura macroura*) and the cuckoos. The mockingbirds, thrashers and the Catbird build bulky structures in vertical crotches of trees and shrubs, but sometimes build on horizontal branches. The grebes, the Black Tern (*Chlidonias niger*), Forster's Tern (*Sterna forsteri*), and occasionally the Common Terns (*Sterna hirundo*), build on floating vegetation. The woodpeckers, titmice, and nuthatches excavate cavities in trees to hold their eggs. Robins and Wood Thrushes build their mud-walled or leaf-mold structures either in upright crotches or saddle them over horizontal forks.

True statant nests either on the ground or above remain in place by the weight and bulk of the materials wedged inside enclosing uprights of vegetation or resting within surrounding walls of earth. It is evident that only those species which attach their nests by means of sufficiently strong fibrous materials or those which build adherent structures are able to use sites which vary from enclosed vertical positions or the broad-based horizontal positions to which the builders of completely statant nests are restricted. Builders of strongly attached nests are able to fasten nests in vertical crotches, with or without foundation materials upon which to rest the bottom of the nest; in horizontal forks, attached at the rim as in the nests of vireos and the Acadian Flycatcher (*Empidonax virescens*); saddled on arching branches as in the Ruby-throated Hummingbird (*Archilochus colubris*) and Blue-gray Gnatcatcher (*Polioptila cereulea*); or on diagonal bases varying at any degree from the horizontal or the vertical positions. Although the birds mentioned in this paper usually build attached nests characteristic for the species, the

combination of site and nest pattern results in variations from the pendulous to the pensile, saddled, and attached-statant types.

Engineering variations in pendulous type nests:

The Baltimore Oriole (*Icterus galbula*) is the best example of a bird which usually constructs nests which are completely suspended from the drooping ends of the branches of larger trees of several species. However, I have found four variations from this position which are apparently as successful as the more typical. Five positions are shown Plate 1: Fig. 1 for the Baltimore Oriole. Typical nest fastened at the drooping ends of branches; Fig. 2. Nest fastened in twigs on the side of a horizontal branch; Fig. 3. Nest fastened at the rim within horizontal fork (vireo-like); Fig. 4. Nest either suspended in a vertical fork or attached to the sides like the nest of the Orchard Oriole (*Icterus spurius*); Fig. 5. Nest attached to small side branches on an upright branch, fastened at both the rim and on one side and either fastened to or resting on twigs immediately below. Actually, these nests represent the almost completely pendulous, and the pensile and attached (pendant) types, variations limited to only a few species of birds of eastern North America.

Engineering variations in pensile type nests:

Pensile type nests are built by ten species of vireos, two species of kinglets, seven species of blackbirds, the Parula Warbler (*Compsothlypsis americana*) and the Acadian Flycatcher. The number of engineering variations likely to most builders of pensile nests apparently is slightly less than for birds which build pendulous nests. Three major factors appear to be involved: 1. A tendency by the builders of pensile nests to utilize weaker and shorter kinds of binding materials because of the scarcity of the longer and stronger materials in woodland habitats; 2. A more limited choice of horizontal forks in trees which meet the basic requirements of the nest pattern; 3. The mode of attaching the nest at the rim. In my experience the species which exhibits the greatest number of engineering variations in this category is the Red-winged Blackbird (*Agelaius phoeniceus*). I have found four departures from the so-called typical nest placement in this species. Fig. 1. The more usual nest is attached at the sides in clumps of cattails, reeds or coarse grasses where the individual stalks are growing close together. Often the bottom of the nest rests in the saddle made by the vegetation as it converges downward, so that it is only partially suspended. Nests built after this fashion on floating mats and in other windswept areas occasionally result in a series of unlined nests superimposed one upon the other, making a total structure a foot or 18 inches in height. These large structures are nearly always found in living cattails or reeds which sway outward with the wind, forcing the attached nest downward along the smooth stalks. Fig. 2. Many nests of the redwing are built in grass hummocks both in marsh areas and in dry fields. Most of these rest solidly

on the tops of the hummocks, enclosed by masses of stalks and are attached firmly at the sides and rims as in the wholly pensile nests. However, some of these nests are poorly attached and occasionally one is not attached at all. Fig. 3. Redwings nesting in upright forks of shrubs in swamps or nearly dry fields usually attach the nests at the sides and rims so that they are partially suspended from two to four uprights like nests of the Orchard Oriole. Fig. 4. A variation from Fig. 3 is accomplished by the builders when they fill the bases of the fork with foundation material upon which the nests rest as in No. 2 while being enclosed by uprights and attached at both rims and sides. This type of redwing nest should be called attached-statant and probably represents the most secure anchorage possible. Fig. 5. Occasionally, redwings build in grape and Virginia creeper vines and in horizontal forks of shrubs. In such situations nests are attached at the rims, forming a pensile type similar to the nests of vireos and the Acadian Flycatcher.

I have found four positions for nests of the Red-eyed Warbling and Yellow-throated Vireos (*Vireo olivaceus*), (*Vireo gilvus*), and (*Vireo flavifrons*), numbered in the order of their prevalence: Fig. 1. Probably more than 90% of the nests of these vireos found are in horizontal end forks where the twigs are usually no more than one-fourth inch in diameter. Nests are built in the narrow ends of the Y-shaped horizontal forks and fastened by wrapping over each prong. These wrappings form the rims of nests and their sole support. Fig. 2. The most common variation from the first position are nests built in U-shaped horizontal crotches formed by two twigs which have grown at right angles to a larger branch two to three inches apart. This position permits the nest to be attached over the rim at the back and two sides. I believe that sites of this type with spacing suitable to vireo nest size are much less common in woodlands than Y-shaped forks at or near the ends of branches, hence, fewer nests are found in this position. Fig. 3. This position differs from No. 1 in that the Y-shaped fork is formed by three branches—one being below the other two—so that the bottom of the nest is supported by the third prong. Nests in this position are of the attached-statant category. Fig. 4. The fourth position is uncommon because the branching habits of most trees at or near the ends of horizontal branches are such that upright forks are not usually formed. A red-eyed Vireo nest, No. 1624 from the Royal Ontario Museum of Zoology, is attached to one upright and one horizontal branch at such an angle that it is pendant. I have found two other nests of this species attached in the same position. Nests of the Acadian Flycatcher exhibit the first three variations shown in nests of the vireos, but apparently, not that shown in Fig. 4. Nests of this species are attached at the rims, but the materials of the basket and sometimes the lining extend beyond the supporting forks so that the structures are partially saddled at both sides. These nests are usually not more than one and one-quarter inches to two inches in total depth as compared with a depth of two and one-half to three inches for nests

of the vireos. The outer diameters of the flycatcher's and varieos' nests all average slightly over two and one-half inches. One of the major functions of the rather stiff lining of all these nests is to serve as inner braceworks which hold the shapes of total structures and in the nests of Acadian Flycatchers serve as cantilevers to support the centers of the nests.

Engineering variations in pendant nests:

The nests of the Phoebe (*Sayornis phoebe*) and Barn Swallow (*Hirundo rustica*) may be : Fig. 1. Pendant (adhesive; Fig. 2. Saddled—adhesive; Fig. 3. Partially statant—adhesive, and Fig. 4. Statant according to the particular site upon which the birds build. All types of variations except, perhaps, that of Fig. 2 are found in cave mouths and under overhanging rock ledges. Originally these species nested entirely in such situations, but now probably nest more commonly on man-made sites. With natural or man-made conditions, nests of the type in Fig. 1 are adherent to broken-and-irregular or slightly downward-sloping vertical surfaces where the mud used by both species holds the nests in place. Top and side views of nests in vertical positions show them to be crescent-shaped and tapered toward the bottom. These nests are usually deeper than nests partially resting on some projection. Nests of the type shown in Fig. 2 are adherent to vertical walls and saddled over insulators, wires, plumbing fixtures or wires and steel rods projecting from the walls beneath concrete bridges. These nests often have the same form as in that of Fig. 1, but are much more securely anchored. The nest illustrated in Fig. 3 is found on the tops of door frames, stripping and other building trim where the weights of nests rest so that they remain more firmly in place than do completely adhesive nests. These nests are not as deep as the two preceding and are more blunt at the bases. Nests of the form shown in Fig. 4 are found on wide-based ledges, rafters, steel braces under bridges, and on shelves placed for these birds by man. The backs of nests are either against or adherent to vertical surfaces but rest on the flat bases as securely as do statant nests. In such situations the original builders in successive nestings over several seasons and/or later builders often amass tall super-structures of as many as ten nests up to a foot in height. The habitat and nesting requirements of these two species often coincide to the extent that either species builds upon the nest of the other.

Engineering variations in attached-saddled nests:

The Ruby-throated Hummingbird, the Blue-gray Gnatcatcher and the Eastern Wood Pewee (*Myiochanes virens*) are probably the best known eastern North American species which build the attached-saddled type of nest. Of these species the hummingbird undoubtedly exhibits the greatest number of adaptations of material to position of nest site. I have found nine different combinations of attachment and saddling in nests of this species. This ability

to saddle successfully such tiny nests over branches often steeply inclined and ranging from one-eighth of an inch to more than one inch in diameter cannot be wholly the result of the small size of the nest and the use of spider silk as a binding material. For the hummingbird also uses glue-like saliva to hold the nest in place. Herrick (1935:157-158) superbly described the various steps followed in nest-building by female hummingbirds. He wrote that "The ruby-throated hummingbird fixes its diminutive and exquisitely wrought nest to a small twig, placing it at a fork, perhaps, or for greater security, extending the base of the nest around the stem, and always building up one side of the nest to compensate for whatever inclination the twig may have. The birds first spread a small wafer of inspissated saliva (Fig. 84) on the chosen twig and, building upon this, literally glue their nest to its support, after the common habit of the swifts. Whether this is an invariable custom in their nest-building or used only when the need is imperative, I cannot say."

Plate 1, Fig. 1 for the hummingbird illustrates a nest sitting astride a one-inch branch with the materials of the nest extending only part way down its sides. Fig. 2 shows a nest in which the wrappings completely encircle a one-quarter-inch twig. Fig. 3 varies from 2 in being attached at the side to a diagonal twig as well as encircling the branch below. Fig. 4 sits astride a larger branch between two uprights to which the nest is attached at the sides. Fig. 5 shows a nest which is saddled on and completely encircles, at the bottom, a diagonal branch. Fig. 6 varies from 5 in being built on so steep a diagonal that both the binding materials at one side and part of the bottom of the nest encircles the twig at such an angle that the nest becomes nearly pendant. Fig. 7 differs from 6 in being saddled over (encircling) a branch at the bottom of the nest and encircling the diagonal as well. Fig. 8, apparently, is found only in trees like the oaks, the smaller branches of which grow in a gnarled and crooked pattern. The nest is deeply saddled and encircles the two diagonals for most of its depth. Fig. 9 closely resembles the placement common to the Eastern Pewees' nests. The nest encircles the fork and its shaft of the horizontal branch with only the outer part of the nest unsupported at the bottom. Nests of the Blue-gray Gnatcatcher and the Eastern Pewee, because of their greater size and lack of the additional feature of glue-fastening characteristic of the hummingbird, show fewer engineering variations. Obviously, the two-inch diameter nests of the Gnatcatcher and the three-inch diameter, flat nests of the Pewee could not be adapted to horizontal branches less than one and one-half inches in diameter for the former and two to two and one-half inches for the latter. Nests of these birds would be inadequate if built on steeply inclined branches of any size unless strongly supported horizontally, because neither bird adequately saddles the branch.

The Blue-gray Gnatcatcher. I have found only three variations in nest posi-

tion for the Gnatcatcher. Fig. 1 shows the commonest position for the nest of this species. The bottom of the nest is saddled over a horizontal branch of an inch or more in diameter and attached to the side of an upright. Fig. 2 shows a nest saddled over a horizontal branch without side support. Fig. 3 shows a nest position which has been reported only a few times. I have found two nests with this placement (Nickell: 159-160), at Aurora, Indiana, and in Oakland County, Michigan. All nests in vertical forks which have been reported have been at low elevations after the fashion of the Alder Flycatcher (*Empidonax traillii*), Yellow Warbler (*Dendroica petechia*) and Goldfinch (*Spinus tristis*).

The Eastern Wood Pewee. The common placement of nests of this species is represented by Fig. 1, for Wood Pewee. The nests are saddled over one or both forks of a horizontal branch and extend back along the shaft. They are attached by outliers of finely shredded bark and plant downs bound in place by spider silk. Fig. 2 illustration shows a saddled nest over a larger horizontal branch attached to an upright branch at the side. Sometimes a nest is found in an upright crotch which is wide enough and sufficiently flat at the base to accommodate a nest (Fig. 3).

ENGINEERING VARIATIONS IN ATTACHED-STATANT NESTS

The three species of Eastern North American birds which demonstrate the greatest variety of engineering features in their nest placements are the Alder Flycatcher, Yellow Warbler, and the Goldfinch. Approximately 7,000 nests of these three species have been collected and examined. Although each species is in a different family, these birds commonly nest in the same types of habitats where available nest sites and nesting materials are basically similar or, in many instances, the same. In particular these birds use the same fibrous materials with which the baskets of their nests are constructed and attached in the nest sites. These birds have overlapping nesting seasons, in southeastern Michigan beginning with the Yellow Warbler in mid-May, the Alder Flycatcher by June 10 and continuing with the Goldfinch by mid-July, ending in late September, and they either gather the bark fibers of the Swamp Milkweed (*Asclepias incarnata*) and other fibrous plants bit by bit, or they commonly dismantle each other's nests to obtain these materials. Moreover, these birds are comparable in size and the nests, which are of the same type, overlap in dimensions to an extent that occasionally birds of each species may superimpose their nests over those of the others. All of the three species show the same variations in engineering features to varying degrees. Fig. 1 is a nest representing the commonest form. The narrow lower part of the vertical crotch is filled with foundation material so that the upper part, attached at the sides, rests upon it. In Fig. 2 the nest is built without foundation so that it is held in place by the attachments at the sides. Fig. 3 shows a

nest built in a horizontal branch with one or two uprights to which the side or sides of the nest are attached. It is usually saddled, also. In Fig. 4 the nest is saddled over a horizontal branch so that the bottom of the nest completely encircles its support. Fig. 5 represents nests encircling inclined stems of shrubs and attached to a side branch. Fig. 6 shows an upright stem to which the nest is attached and a lateral branch which is saddled and encircled by the bottom of the nest. Fig. 7 shows a nest attached at the rim to a lateral branch wrapped at one side to an upright stem, making a pendant form. In Fig. 8 the nest is attached at the sides between two uprights and is unsupported at the bottom. In Fig. 9 the nest is attached at the rim to a horizontal fork so that it forms a pensile type like the nests of vireos. I have seen the nests of both the Yellow Warbler and the Alder Flycatcher built in nests of Red-eyed Vireos.

ENGINEERING VARIATIONS IN SOME STATANT NESTS

The Robin (*Turdus migratorius*) and the Wood Thrush (*Hylocichla mustelina*) usually build statant nests which are unattached to their sites. The rigid nest cups of Robins' nests are usually built of heavy mud, occasionally of muck or leaf-mold. Wood Thrush nests cups usually contain only the lighter weight leaf-mold or muck. I have found four engineering aspects in the nest of these species. Fig. 1 illustrates the most abundant form of the nests of the Wood Thrush and the Robin in wild nature. The Wood Thrush is still largely restricted to its usual habitat, but the Robin in modern times has adapted to man-made situations so that its most abundant nest sites are probably represented in Fig. 4. In Fig. 1 the nest is in upright crotches of trees or the larger shrubs, enclosed by two to four uprights. Nests rest solidly in the bottoms of the crotches by their own weight and often partially saddle the uprights in narrower crotches. Fig. 2 shows nests built over horizontal crotches and saddled. Fig. 3 shows a nest found on branches which droop to form inclines. Nests in this position are thickened on the lower sides by built-up foundation materials which permit the nest to stand in a level position. Fig. 4 shows the nest of the Robin around human habitations. This type of nest is found on window ledges, rafters, steps, niches in walls and chimneys, nesting shelves, transformer boxes, tops of fence posts and on brace flanges under bridges.

THE BRANCHING HABITS OF TREES, SHRUBS AND FORBS AS FACTORS IN BIRD NEST SITE CHOICE

Most species of birds which nest above ground must select sites in some kind of vegetation which afford them opportunity to build their characteristic types of nests. That birds do select, within the framework of instinct, the

sites on which they build their nests, cannot be doubted because of the high percentage of nests which are successfully placed to fulfill their functions as compared with those which are failures because of faulty construction or anchorage. There is evidence in the pre-nesting behavior of many species of birds that some degree of experimentation is followed before a final choice of site is made. In some cases the male birds which do not later participate in nest construction, may either choose the nest site or influence the female in her choice. The male robin's molding activity on or in a variety of crotches or bare branches, some completely unsuitable for robin nest placement, is practically identical with the activity of the female when pressing mud in molding the well-formed nest cup. The male catbird carries twigs to one or more crotches while being followed closely by the female. Later, the female, may accept his choice and build upon his beginnings, or choose another site leaving the forgotten twigs unused. Further evidence of experimentation is suggested by frames found on different sites built by several species of birds before an actual nest is completed. I have observed this behavior in the Red-eyed Vireo, Red-winged Blackbird, Yellow Warbler, Barn Swallow, Phoebe, Goldfinch, Cardinal (*Richmondena cardinalis*), and Catbird (*Dumetella carolinensis*). A considerable percentage of these abortive attempts at nest building may be due to the birds having first chosen sites which are unsuitable for their types of nests and their modes of attachment. Several nest frames I have examined have been in forms too narrow to accommodate nests of the dimensions required by the birds. Other sites of abortive nesting attempts may have failed to satisfy the instinctive requirements for stability. Field studies I have made of the species of vegetation in which several species of birds have nested in the Southeastern Michigan Region during the last 20 years have shown that three aspects of vegetation appear to be important factors for nest sites and nesting materials of birds: 1. The branching habits and other characters of individual plant species; 2. Abundance, distribution and growth stage, and 3. Availability of plants with bark, fibers, twigs, rootlets, leaves and downs suitable for nest construction. I have found that some species of trees and shrubs are almost never used as nest sites by any species of birds in this region while others are used abundantly by several species. Good examples of trees and shrubs which are not commonly used as nest sites by birds are sumacs, poplars, ashes, hickories, wild cherries, birches, and others, probably because of open branching habits, smoothness of bark, brittleness of twigs and other characters which render them largely unsuitable for secure nest anchorage for most birds.

Staghorn Sumac. One of the most abundant and widely distributed shrubs of this area is Staghorn Sumac (*Rhus typhina*), yet it is not often used as nest sites by any species of bird, and wherever it is used, then only when individual branching arrangements depart from the usual growth habit or are supplemented by several species of grapes, Virginia Creeper (*Parthenocissus*

quinquefolia) and other vines which are twined around them. The parallel growth of the upright terminal branches furnish no enclosing crotches to support statant nests of Catbirds, Brown Thrashers, Robins, Cedar Waxwings (*Bombycilla cedrorum*), and Kingbirds (*Tyrannus tyrannus*) although the habitat is suitable, as indicated by nests in other species of shrubs and trees growing in the midst of Sumac colonies. Occasionally, a Goldfinch will build a nest in a Sumac shrub which has a two-pronged, Y-shaped, upright terminal sufficiently wide to accommodate its nest, but only because the structure is securely attached at two sides and wedged in the bottom of the fork. In a 20-year study (1934-1953) of the Catbird, I found 3,939 nests which were built in 116 species of trees and shrubs of which 54% were in the first five species, all shrubs or low much-branched trees, listed in Table 2. Only seven nests were found in Staghorn Sumac and all were supplemented by vines climbing on the sumac. In a six-year Goldfinch study (1950-1955) 4,084 nests were found in 80 species of trees and shrubs. Over 74% of these nests were in the first five species (Table 2), also, all shrub or shrub-like. Twenty nests were in Staghorn Sumac (Table 1).

Quaking Aspen. Quaking Aspen like Staghorn Sumac is not often used by tree-nesting birds, yet it is abundant and widely distributed in both wet and dry situations. The irregular distribution of branches along the trunks, the scarcity of enclosing uprights, the smoothness of its bark on both trunk and branches, the brittleness of its small twigs and its lack of foliage density all appear to render it unfit for nest sites for most birds which build completely statant nests. As abundant as it is in the generalized habitats of Catbirds, I have found only five of 3,939 of these statant nests in its branches in 20 years. Again, as in Staghorn Sumac, the Goldfinch was able to use the Quaking Aspen because of its smaller attached nest. One hundred and fifty-seven (3.8%) of 4,084 nests of the Goldfinch found in six years were in Aspen, (Table 1). However, the Goldfinch is the only species which more than rarely uses Aspen for nest sites in the southern Michigan region. Table 1 lists eight species of trees and one shrub which are distributed over the habitats of the Goldfinch and the Catbird in numbers which should place them among those more commonly used as nest sites. A comparison with Table 2 shows them to be much less used than one might expect considering their commonness. The structure of these plants render them less suitable as nesting sites.

A comparison of the numbers of attached-statant nests of the Goldfinch and the statant nests of the Catbird found in eight common species of shrubs in Southeastern Michigan is shown in Table 2. Both the Goldfinch and the Catbird are among the most abundant nesting birds in Southeastern Michigan as the 8,023 records for the two species show. Both species exhibit a considerable degree of tolerance in habitats which they will occupy, and these habitats overlap at many points.

Gray Dogwood. It will be noted in Table 2 that Gray Dogwood as a nest-

ing shrub occupied first place for both species of birds in their over-all habitats. Gray Dogwood has the same rank as a valuable shrub for nesting four other species of birds also. These species are the Yellow Warbler, Alder Flycatcher, Cardinal, and Field Sparrow (*Spizella pusilla*), all common species, but with somewhat more restricted habitats than the two preceding. Gray Dogwood and the Hawthorns are undoubtedly among the most abundant and widely distributed shrubs in Southeastern Michigan and Southwestern Ontario. The habitats of both show considerable overlapping largely, apparently, because of the tolerance of Gray Dogwood for both wet and dry situations. Billington (1949:243) stated that, "The Panicled Dogwood is one of our most common shrubs. It grows abundantly along the roadsides and in fence rows bordering our fields and woods. It grows on the banks of streams, and everywhere it makes a beautiful appearance when in flower."

TABLE 1

FREQUENCY OF USE OF NEST SITES IN NINE SPECIES OF TREES AND SHRUBS BY THE GOLDFINCH (304 nests) AND THE CATBIRD (28 nests) COMPARED TO EIGHT MORE COMMONLY USED TREES AND SHRUBS LISTED IN TABLE 2.

PLANT SPECIES	Goldfinch Number of Nests	Catbird Number of Nests
Quaking Aspen (*Populus tremuloides*)	157	5
Wild Black Cherry (*Prunis serotina*)	49	9
Cherry Birch (*Betula lenta*)	20	0
Staghorn Sumac (*Rhus typhina*)	20	7
White Ash (*Fraxinus americana*)	19	0
Choke Cherry (*Prunus virginiana*)	13	3
Black Ash (*Fraxinus nigra*)	13	0
Paper Birch (*Betula papyrifera*)	11	1
Shagbark Hickory (*Hicoria ovata*)	2	3

The branching habit of Gray Dogwood, when thinly distributed in wet or dry situations, and at the edges of dense thickets of this species, is near optimum for nest sites of several species of our small birds. Its upright branching pattern, with terminals somewhat evenly distributed around each central axis, furnishes two to eight (average four) points of attachment for attached-statant nests and enclosing baskets for statant nests. Reference to Table 2 will show that Goldfinches using Gray Dogwood built about one nest in a horizontal position to every 172 nests built in vertical crotches. The greater number of Catbird nests in horizontal positions (about one to 20 in vertical

TABLE 2

COMPARISON OF EIGHT GENERA AND SPECIES OF TREES AND SHRUBS AS SITES OF 5,491 NESTS OF THE GOLDFINCH AND THE CATBIRD WITH EMPHASIS ON THE FIRST FIVE UNDER EACH SPECIES OF BIRD

GOLDFINCH—PLANT SPECIES	No. of Nests	Nos. in horizontal positions	Nos. in vertical positions	Percentage in each species	Average height in feet above ground
1. Gray Dogwood (*Cornus racemosa*)	1203	7	1196	29	4.6
2. Hawthorns (*Crataegus* spp.)	579	92	487	14	5.4
3. Shrub Willows (*Salix* spp.)	529	7	522	12.9	6.2
4. Red Osier Dogwood (*Cornus stolofera*)	450	3	447	11	4.2
5. Elms (*Ulmus* spp.)	266	33	233	6.5	6.6
6. American Elder (*Sambucus canadensis*)	89	4	85	2.1	4.8
7. Wild Grapes (*Vitis* spp.)	14	3	11	.34	5.6
8. Tartarian Honeysuckle (*Lonicera tartarica*)	9	0	9	.22	5.8
CATBIRD—PLANT SPECIES					
1. Gray Dogwood	581	29	552	14.8	5.2
2. Tartarian Honeysuckle	543	143	400	13.8	5.4
3. Hawthorns	448	28	420	11.3	5.4
4. Wild Grapes	326	114	212	8.2	6.0
5. American Elder	229	32	197	5.8	4.6
6. Red Osier Dogwood	99	27	72	2.5	4.6
7. Shrub Willows	94	6	88	2.4	4.8
8. Elms	32	3	29	0.8	5.4

positions) is largely explained by the tendency of this bird to nest in denser growth where crossing horizontal branches and vines form the hammock-like sites used.

The Hawthorns. Although the Hawthorns have a considerably different branching pattern from the Gray Dogwood, they are nevertheless as suitable for nest sites. The general arrangement of Hawthorn branches tends to be a combination of upright and horizontal growth with interlacing diagonals and cross-branches which furnish many good sites in either vertical or horizontal positions. The ratios of horizontal positions to vertical positions used by the Goldfinch and the Catbirds in the Hawthorns are about 1.2 to 6.3 and 1 to 16 (Table 2). The greater proportion of Catbird nests in vertical positions is explained by this bird's tendency to build near the centers of shrubs in the main trunk crotches while Goldfinches more often choose smaller shrubs and the outer parts of the larger shrubs. The number of branches forming crotches for nest sites average 3.3 per shrub.

Tartarian Honeysuckle. Tartarian Honeysuckle is a shrub which was introduced into Southeastern Michigan for landscaping large estates, probably 50 years ago. It forms dense hedges along roads, paths, woods edges, and beside buildings and old stone walls, most favorable habitats for Catbirds but scarcely used by Goldfinches. Goldfinches use Tartarian Honeysuckle when it occurs as scattered shrubs in open areas where its seeds have been dropped by birds. The high rank it occupies as a nesting shrub for Catbirds is due to its great suitability and abundance in the area where my studies were most intensive. Its growth pattern consisting of up to eight upright terminal branches in the younger growths and its habit of falling to a nearly recumbent position from the center of clumps when it is mature, creates both horizontal and vertical sites for Robins, Brown Thrashers, Catbirds, Cuckoos, Mourning Doves and Song Sparrows (*Melospiza melodia*). The ratio of 1.3 nests in horizontal positions to 3.8 nests in vertical positions for nests of the Catbird is probably one of the closest approximations to even distribution between the two positions found in any shrub or tree which is commonly used as nest sites. Uprights around the terminals or the central stems average 4 per shrub.

Shrub Willows. Several species of Shrub Willows occupying third place for nest sites of the Goldfinch have branching patterns similar to that of Gray Dogwood. The habitats of these shrubs, however, are confined chiefly to wetter situations which are not commonly chosen by the Catbird for nesting. Moreover, the upright crotches of the majority of these shrubs are narrow, hence more suitable for the smaller, attached nests of Goldfinches than for the much larger, unattached nests of Catbirds. Goldfinch nests were 5.6 times as numerous as nests of Catbirds in these shrubs and averaged over one foot higher above ground. Good horizontal sites are not common in Shrub Willows.

Red Osier Dogwood. This species is a native shrub found generally in marshes, bogs, along streams and other wet situations. This limitation gives it a status comparable to that of the Shrub Willows as nest sites for both the Goldfinch and the Catbird. Upright terminals forming the crotch average 3.1, but are usually evenly spaced. Its growth in the more open situations renders it more suitable for Goldfinches than for Catbirds.

The Elms. The majority of the Elms used as nest sites by the Goldfinch and the Catbird are saplings which are three to 35 feet in height. Nearly all Goldfinch nests are placed in the top trunk forks of smaller trees or in tufts along the horizontal branches of larger saplings. Practically all Catbird nests are placed in the lower main trunk crotches of saplings of medium size, as few horizontal elm branches are suitable for holding their statant nests. These crotches average 3.6 uprights and 5.4 feet above the earth. The average height of nests of the Goldfinch was over one foot higher (6.6 feet) because of their greater number of high nests in horizontal positions (Table 2).

American Elder. This shrub is common in moist situations where both the Goldfinch and the Catbird nest. Its habitat limitations confine its growth largely to stream banks, flood plains, and edges of bogs and marshes so that it is not nearly as widely distributed as most of the foregoing species. However, its branching pattern renders it moderately suitable for nest sites for several species of birds. This branching pattern is loose and open so that many of its horizontal branches are unsuitable as sites for either attached or statant nests unless supplemented by branches of other vegetation. The central crotches, formed by an average of four branches, are flaring so that they are usually more adaptable to the larger nests than to the smaller nests of Goldfinches, Alder Flycatchers, and Yellow Warblers, although these smaller birds, which attach their nests, do find a moderate number of crotches which fit their needs. The ratio of horizontal positions used by the Goldfinch and the Catbird in 282 nests in this shrub was 1:8. The ratio of horizontal sites used by the Goldfinch to vertical sites was about 1 to 21, while the Catbird's ratio of horizontal sites used to vertical sites was 1 to 7. The number of Catbirds' nests found in Elder was two and one-half times that of the Goldfinch. The average heights of nests above the ground was almost the same: 4.8 feet for the Goldfinch and 4.6 feet for the Catbird.

The Wild Grapes. The various species of wild grapes of this region grow mostly in moist or dry thickets and along fence rows where they attach themselves to other, supporting vegetation. They are found only sparsely in the more open areas in which Goldfinches most commonly nest, but are common in one of the more favored types of Catbird habitat. The interlacing network of crossing branches and tendrils which these shrubs form on shrubs, trees and fences, constitute good horizontal sites and the three (or four) upright terminals of smaller vines are arranged around the central axes, producing basket-like vertical sites. The ratio of Goldfinch nests to Catbird nests found

in the grapes was 1 to 23. The ratio of nests of both species found in horizontal sites to those found in vertical sites was 1 to 2.

Herbaceous Plants. Forbs are not often used as nest sites by most species of birds which nest above the ground. Most of these plants are too weak to support the weights of nests except in clusters as in the nests of Long-billed Marsh Wrens (*Telmatodytes palustris*), Redwings and Least Bitterns (*Ixobrychus exilis exilis*) which attach their nests to cattails, reeds and other herbaceous marsh vegetation. Moreover, most larger forbs which could be used if suitably branched and sufficiently strong to support nests, have not reached their full growth by the time many birds are at the height of their nesting activities. A notable exception is the Goldfinch which begins its nesting about mid-July and nests again about mid-August. I have found 15 nests of this bird in Canada Goldenrod (*Solidago canadensis*) and two nests in Swamp Milkweed. Others have reported nests of this species in thistles. I have found one nest of the Catbird in Tall Meadow Rue (*Thalictrum polygamum*).

PLANT AND OTHER MATERIALS USED IN BIRDS' NESTS

The nesting materials of birds are usually found in the immediate vicinity of the nest site and their variety, when considered as a whole for all species nesting in any region, is almost as great as the plant species themselves. In addition such materials as mud, hair, feathers, spider silk, dried spittle of spittle bugs (Family Cercopidae), and man-made materials of many kinds are included in all parts of nests.

Again, as in choosing nest sites, birds instinctively select from their environment materials which fit the need of their own kinds of nests. The Baltimore Oriole usually nests in the vicinity of streams, lakes, swamps, and other wet situations where Swamp Milkweed grows in greater or lesser abundance. Its pendulous nest frame is usually built of the milkweed's outer bark fibers which I have many times seen them gathering. If cotton wrapping-twine, yarn, or other strong, fibrous and flexible materials approximating the qualities of the natural material is found, they will be used. The lining of the nests is of the long hair of cattle and horses, or finely shredded, spring-like bark of grapes and other vines. The apparent function of the linings in these nests is primarily to prevent their collapse from the weight of the adults, eggs, and young, as no appreciable amount of insulating materials is used. Obviously, the Baltimore Oriole would find it impossible to suspend its nest from slender branch ends if it attempted to make use of twigs, leaves, coarse tree bark, stiff weed stalks, tendrils, moss, mud, and many other materials used by several species of birds building statant nests. Just as obviously, the Catbird, Robin, Phoebe, or Hummingbird would fail if they should attempt to duplicate the Orioles' feat of engineering with the kinds

of materials which they instinctively gather from their surroundings. Hence, each species must select the kinds of materials which meet the requirements of its nest pattern. Among birds building different types of nests and following different patterns of construction there are many points of similarity in the use of materials, but most birds indicate one or more points of specificity in selecting certain materials or good substitutes for them in constructing their nests. The Baltimore Oriole, Red-winged Blackbird, Yellow Warbler, Redstart, Alder Flycatcher, Goldfinch, Cedar Waxwing, Kingbird, and sometimes the Catbird, Phoebe, Red-eyed Vireo, Acadian Flycatcher, and others, representing pendulous, pensile, pendant, adherent, attached, and statant nest types will all use the outer bark fibers of Swamp Milkweed in their nests if it is abundant near at hand or if it can be obtained easily from abandoned nests of other species. This material is used as binding for other short loose materials and for the attachment of nests. Only the first eight above listed species use milkweed fibers regularly; the others make use of it, apparently, when it is easily obtained or when other materials are not available near the nest site. Milkweed fibers in wild nature far from human homes are often almost the only available material with sufficient strength, length, and flexibility with which the Baltimore Oriole could build a secure, suspended nest. In this sense this material, then, becomes close to being specific for this bird. The other users of Swamp Milkweed fibers build their nests in such situations as enclosed forks or resting securely on foundations so that this material is useful to various degrees but becomes less than specific because they can and do use the shorter, weaker, and less easily obtained fibers of common milkweed (*Asclepias syriaca*) and other weeds, and a variety of plant downs and catkins which can be felted sufficiently to hold nests together.

Other apparent examples of specificity in nesting materials are found in the use of spider silk as a binder and a substance of attachment by the vireos, Eastern Wood Pewee, Blue-gray Gnatcatcher, and Ruby-throated Hummingbird. Due to their methods of saddling, encircling and attaching nests and the need for a fine but strong material which is commonly found in their habitats, probably no other type of substance will quite suffice. Another desirable quality of spider silk in both the attachment of the nests to sites and the fastening of lichens with which these nests are covered, is its stickiness when wet by the birds' saliva at the time it is used. Bits of lichens used on the outer walls of the nests of pewees, gnatcatchers, hummingbirds, Black-capped Vireos (*Vireo atricapillus*) and the Yellow-throated Vireo are held in place by these apparently irreplaceable spiders' silks. For the Robin, Barn Swallow, and Phoebe mud becomes a specific building material for which, apparently, there is no adequate substitute. The use of mosses by the Phoebe appears to be another example of specificity but examination of large numbers of nests in relation to the availability of this material shows that weed barks and other fibrous materials are used frequently to act as binders for the

mud used in nests. Rootlets used as nest linings by Catbirds and Brown Thrashers appear to be the most constant feature of their nests. The daily familiarity of these birds with the rootlets of trees and shrubs which they uncover in feeding in thickets and on the forest floor may be a factor in their use. These rootlets which are moist and flexible when placed in the nests become like small wire springs when dry. In this dry state they serve as an inner bracework which preserves the shape of the nest baskets. In the final analysis it may be said that engineering variations found in the nests of some birds are the result of instinctive nest patterns in species which are adapted to mechanical variations in nest sites within the limitations imposed by nesting materials.

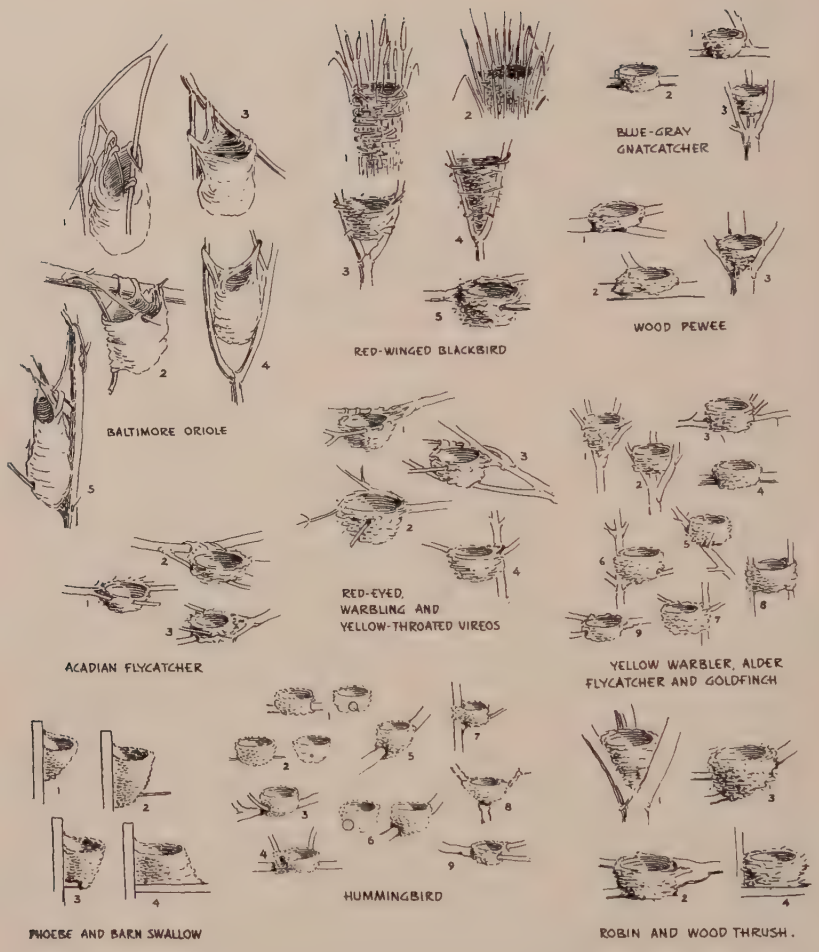
SUMMARY

During 35 years of examining in the field and collecting about 20,000 nests of 169 species of birds, I have collected a great mass of data on the nest sites and nesting materials of these birds and have noted the engineering variations from the so-called typical which have occurred for each species. My studies in the region of Eastern North America between 50° and 30° north latitude and between the 100th meridian and the Atlantic Ocean have indicated that only about seven per cent of the species listed in Eastern North America exhibit any great degree of variation from their usual nest placements.

Important factors involved in nest placement, which demonstrates engineering variations which I have found, occur in nests which are built above ground and all except a few have occurred in nests which are attached or adherent to their supports. Engineering variations are listed for the nests of 16 species of birds which are shown on Plate 1 accompanying the text. The types of nests listed as showing these variations are pendulous, pensile, pendant, attached-saddled, attached-statant, and statant.

The branching habits of trees, shrubs and forbs are discussed in relation to nest site choice. Table 1 lists nine species of trees and shrubs which are common but not frequently used as nest sites and compares the ferquency of their use by the Goldfinch which builds attached-statant nests, and that of the Catbird which constructs statant nests. Also, this table compares the use of these shrubs with the frequency of use of eight other plant species which are most frequently used as nest sites in southeastern Michigan (Table 2). Table 2 lists eight species of trees, the first five of which under the Goldfinch and the Catbird show the greatest usage in 4,084 nests of the former and 3,939 nests of the latter. The abundance, distribution and usability of the various species listed is discussed in the text.

Forbs are shown to be largely unsuitable for nest sites for most species of



ENGINEERING FEATURES OF NESTS OF 16 BIRD SPECIES

birds because of not being available at the height of the nesting season and by being poorly branched and weak. Goldenrods, thistles, cattails, reeds, and marsh grasses are exceptions for the Red-winged Blackbird and the Goldfinch.

Nesting materials are discussed, their types and the specificity of their use by the bird species indicated. Some apparent specificity in the use of certain materials is shown to be only apparent, because of known substitutions which some species have made successfully.

Illustrations of Engineering Variations by Luella C. Schroeder

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THE LIFE HISTORY OF THE FRESHWATER RED ALGA *TUOMEYA FLUVIATILIS* HARV.[1,2]

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INTRODUCTION

Of the several genera of freshwater Rhodophyta belonging to the subclass Florideae, *Tuomeya fluviatilis* Harv. is probably one of the rarest forms. In North America there are five genera of freshwater Florideae: namely, *Chantransia* (*Audouinella* in Smith, 1950), *Batrachospermum, Thorea, Lemanea* and *Tuomeya.*

Tuomeya was first found in Alabama by Professor Tuomey about 1857 and at about the same time near Fredericksburg, Virginia, by J. W. Bailey. Tuomey (1858) sent some dried specimens of the alga to W. H. Harvey who named and described it in volume three of *Nereis Boreali Americana* published in 1858. Kuetzing also described it from specimens presumably sent him by Bailey, and named the plant *Baileya americana.* The generic name *Baileya,* however, had already been applied to a genus of Compositae by Asa Gray (1848) and for that reason the name *Tuomeya* has been allowed to stand.

In North America, the present known range of *Tuomeya fluviatilis* Harv. extends from the Laurentian mountains of Quebec to Louisiana. In 1933 I found new localities for the alga in Pennsylvania, in Mountain Creek, and Tom's Run Creek in the village of Pine Grove Furnace, Cumberland County, in the Michaux State Forest. Plants in Mountain Creek were growing in great abundance on rocks about a foot below the surface of the water of this rapidly flowing stream.

The general structural features of *Tuomeya* resemble somewhat those of Batrachospermum. In plants of *Tuomeya* however, the frond is much more compact, and the whole body is so much more rigid that when plants are removed from the water, they retain their form and do not collapse into a gelatinous mass. Specimens when dried become quite hard and brittle.

Tuomeya is a bushy plant composed of branching, cylindrical fronds

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[1] This paper is based on a dissertation submitted as partial fulfillment of the requirements for the degree of Doctor of Philosophy from the Johns Hopkins University.

[2] Additional information obtained from further study of this plant has been included in this paper.

which taper at their tips. It usually grows from 1 to 5 cm. high but may occasionally attain a height of 6.5 cm. (Plate 1, Fig. 1). The adult frond is uniaxial, but from each cell of the axis a whorl of lateral assimilative branches grows. The compact growth of the lateral branches gives *Tuomeya* its characteristic rigidity (Plate 2, Figs. 13-16).

Setchell (1890) in his study of Tuomeya made a comparison of *Tuomeya* with *Batrachospermum* and *Lemanea*. Setchell was unable to make clear the development after fertilization and the formation and fate of the carpospore of *Tuomeya*. The formation of a chantransioid or juvenile stage was hinted by him, but he did not illustrate any such structures. For this reason, it seemed appropriate to try to settle some of the unknown and doubtful phases of the life cycle.

After considerable study it was found that Setchell had misinterpreted the function of certain structures: in confusing carpospores for spermatia (antheridia), Setchell was unable to ascertain the formation, structure, and fate of the carpospore. Because of this a re-investigation of the life history was undertaken.

METHODS

The specimens used in this study were determined as *Tuomeya fluviatilis* Harv. by Professor Wm. Randolph Taylor and by Dr. Marshall Howe then director of the New York Botanical Garden.

Both living and preserved plants were used. Those to be sectioned were usually fixed in the field or in the laboratory shortly after collection. The following fixing agents were used: Flemming's solution weak and strong mixtures; various chromic acid mixtures; formol-acetic-alcohol; Juel's mixture of zinc chloride, alcohol, and acetic acid; and Carnoy's fluid. Of these the Flemming's weaker solution, formol-acetic-alcohol and Juel's mixture appeared to give the best results.

Dehydration was accomplished in one of three ways: namely, by ethyl alcohol, by tertiary butyl alcohol or by dioxane. Little difference could be noted between the ethyl alcohol and tertiary butyl alcohol, since both gave good results; but not much success was obtained using dioxane, although it was not used to the same extent as were the preceding two dehydrating agents.

Material dehydrated in ethyl alcohol was cleared with xylene or chloroform. No clearing agent was necessary when the other two dehydrating agents were used. Paraffin infiltration was begun after pure tertiary butyl alcohol or dioxane dehydration.

Sections were stained with Haidenhain's iron hematoxylin method. Other stains employed were iodine and crystal violet, safranin and fast green and basic fuchsin for the Feulgen nucleic acid reaction. Best results were obtained

with iron hematoxylin and iodine-crystal violet. The Feulgen reaction did not appear to be specific for the nuclei of *Tuomeya*. Fresh portions of the plant crushed out on slides were sometimes stained with Mayer's haem-alum stain for general observations, and also the aceto-carmine smear technique was used.

VEGETATIVE STRUCTURE

The carpospore of *Tuomeya* after germination, forms a juvenile plant called the chantransioid stage, which is quite different from the adult shoot into which it develops.

The carpospore after escaping from the carposporangium rounds up forming a globular body. The carpospore may germinate within a few days after being discharged and the young germling (Plate 1, Fig. 2) looks much like those Kylin (1916a.), (1917) has shown for *Nemalion multifidum* and *Batrachospermum moniliforme*. Frequently, with plants growing in aquaria, the carpospore germinates into a filament several cells long while still attached to the parent plant (Plate 1, Fig. 3).

At germination a papilla is formed on the wall of the spore, and the protoplasm flows out into it while it is elongating. Cross walls appear after the protuberance reaches a length of three to four times the diameter of the spore wall (Plate 1, Figs. 4-5). In silica gel cultures, carpospores germinated after a few days and grew to about twenty cells in length, but the germlings did not thrive well. It was evident that no rhizoid-like outgrowths were formed on the cell next to the old spore wall as Kylin (1917) has shown for *Batrachospermum*. The carpospore wall soon falls away leaving a filament of cylindrical cells each about four to six times as long as it is broad (Plate 1, Figs. 5, 6). Each cell, except the basal one, possesses a parietal chromatophore which is somewhat band-shaped. The filaments may branch, although they are usually composed of several cells before branching occurs.

Young plants scraped from the rocks have supplied the only other source of material for the study of juvenile stages. These appear as small dark green humps on the rocks, to which they adhere tenaciously. These humps or "turfs," referred to by Setchell as the possible juvenile stage, may be one to four millimeters in diameter and consist of basal heavy-walled cylindrical cells (with granular cytoplasm and poorly developed chromatophores) united into filaments that branch and intertwine repeatedly. They give rise to erect filaments which are sometimes branched but usually are simple and composed of six to eight more or less monilform cells (Plate 1, Fig. 7). The apical cell of the erect filament is swollen into a monosporangium (Plate 1, Figs. 7, 8) with dense cytoplasm, from which a spore is liberated by rupture of the monosporangium wall. A new sporangium proliferates on the same filament growing up within the ruptured wall of the previous one while

often several old walls may be seen still adhering to the base of the new sporangium (Plate 1, Fig. 8).

The basal filaments of the juvenile plant give rise to the sexual shoots of *Tuomeya* (Plate 1, Figs. 9, 10). The first sign of this formation is observed when a short apical cell is formed instead of the usual long cylindrical cell. This is destined to be the growing point of the sexual frond. By the activity of this apical cell short sub-apical cells are produced on which lateral branch initials arise (Plate 1, Figs 9, 10, 11). The development of these lateral outgrowths or branch initials is essentially the same as that which takes place on the mature plant except that, at the time the frond is initiated, the cells have extremely thick walls, they are less compact, and the chromatophores are poorly developed. As growth and division take place, the sexual branch pushes up above the juvenile plant.

DEVELOPMENT OF THE MATURE PLANT

The adult plant develops by means of a dome-shaped apical cell which cuts off short cells that at first average 1.5 to 2.0 microns long and 9.0 to 14 microns in diameter (Plate 2, Fig. 12). These give rise by considerable elongation to the large axial cells (Plate 2, Fig. 16) which in older parts of the frond may attain a length of over 200 microns and a diameter at the nodes of over 28 microns (Plate 2, Fig. 13).

In general outline these axial cells are more or less cylindrical, but they are larger at the base than at the apex (Plate 2, Figs. 13, 14). The cytoplasm appears reticulate in young fronds (Plate 2, Fig. 14). In comparison with the size of the cell, the nucleus is very small but can sometimes be seen in fresh material when crushed out on a slide. In stained sections the ovoid nucleus is shown to consist of a faintly staining nuclear plasma surrounding a darkly staining nucleolus.

Usually three or four cells below the apical cell, lateral branch initials arise from the axial cells (Plates 1, 2, Figs. 11, 12) and at first are papilla-like. They increase in size and become cut off from the mother cell by vertical cross walls. Usually six such cells form a whorl around each node of the central axis (Plate 2, Fig 15). These initials usually give rise at their distal end to two or three separate cells, each of which in turn gives rise to two or three cells and so on until ultimately there is formed a much forked lateral branch (Plate 2, Figs. 13, 16).

The young axial cells continue to elongate greatly whereas the lateral branch initials do not; but by the increase in size of all the cells, branch initials come in contact with the base of the axial cell above so that in older parts of the frond they appear to arise from two axial cells (Plate 2, Fig. 16).

When mature, the frond appears to be made up of an inner and an outer stratum, the inner composed of large cortical cells, the outer of smaller cells

(Plate 2, Fig. 16). The outer cells however are not united as in *Lemanea,* into a rigid parenchymatous tissue; although in old portions of the frond they are closely apposed. Each cell contains a parietal chromatophore which in the outer assimilative cells may obscure the other cell contents. The chromatophore of the inner cortical cells is less conspicuous and is composed of several disc-shaped bodies connected by a thin thread-like portion while in others it is ribbon-like.

The cells of the inner stratum increase in size as the frond grows and when mature are five to six times as large as when initiated. The cytoplasm appears to be hyaline with the exception of bright refractive granules which sometimes occur in large numbers and which turn red when treated with iodine. It is possible that the large cells of the cortex are used for storage and these granules are a reserve food.

The assimilative cells are much smaller than cells of the inner stratum (Plate 2, Fig. 16). They form a layer one to three cells thick; the outermost cells are smallest and ovoid in shape while the inner are somewhat ellipsoidal, usually twice as long as broad. A large parietal chromatophore makes it difficult to see the small nucleus except in sectioned material. A few refractive granules may occur in these cells also.

From the base of the branch initial cell, corticating filaments grow downward around the central axis (Plate 2, Figs. 16, 17). These simple, rarely branched filaments, consist of cylindrical, thick-walled cells, about four times as long as broad, with thread-like or ribbon-like chromatophores. They may traverse the axis for several nodes and completely obscure it in older portions of the plant, filling the space between the internodes and the lateral branches so that the frond appears to be a solid structure.

In longitudinal and cross sections, assimilative branches spread out tree-like from the node and join others at the periphery thus forming a cylindrical frond (Plate 2, Figs. 13, 15, 16).

One-celled projections which have been called hair cells may occur at the tips of assimilative cells. These are composed of a swollen basal part which narrows to a fine hair. The cytoplasm is granular and no chromatophore is present. The nucleus is small, occurring either in the swollen part or often in the hair.

The function of the hair cells has not been ascertained; but I noticed that they developed much more extensively on plants kept in aquaria than on plants in their natural habitat.

DEVELOPMENT OF THE SPERMATIUM

Tuomeya produces spermatangia at the tips of lateral branches. The spermatangium mother-cell cannot at first be distinguished from an ordinary assimilatory cell (Plate 3, Fig. 18); it is ovoid, but as successive spermatangia are produced on the mother-cell it becomes more or less heart-shaped (Plate

3, Fig. 19). There is a well-developed chromatophore at the distal end, under which the nucleus lies somewhat hidden, and large refractive granules occur in the cytoplasm.

The spermatangia are small, ovoid cells somewhat flattened where they are joined to the mother-cells (Plate 3, Fig. 18). They grow out successively from the mother-cells one or two at a time and are then cut off from them by cross walls. They attain a size of 3.5 to 4.0 microns. At first they each possess a parietal chromatophore (Plate 3, Figs. 20, 21) which later appears to break down. This may be seen in sections stained with Haidenhain's iron hematoxylin where the chromatophore does not take a rich bluish stain as the normal chromatophore of an assimilative cell does but appears rather a brownish color. In the mature cell only a small remnant of the chromatophore remains. In the spermatangium of *Tuomeya* the cytoplasm appears reticulate. The nucleus is small and in young cells appears to be in an interphase stage with a prominent nucleus surrounded by a more or less clear plasma (Plate 3, Fig. 21). As the cell matures, the nucleus undergoes changes; it now appears to be in a prophase stage showing usually 7 to 9 granules (Plate 3, Figs. 18, 22).

The spermatium is discharged as what appears to be a naked, non-motile protoplast, by the rupture of the distal end of the spermatangium wall. It is globular in shape at this time and possesses a single, small nucleus which still appears to be in a prophase stage. Shortly after discharge the protoplast forms a wall around itself which is clearly visible when the spermatium becomes attached to the trichogyne. The cytoplasm of the cell is reticulate and rather granular, and only part of the old chromatophore is apparent as a small globule. The chromatophore stains poorly with iron hematoxylin, appearing as a yellow or orange body, and is not apparent in spermatia attached to the trichogyne.

In discharged spermatia I have found what appears to be a mitotic division of the nucleus (Plate 3, Fig. 23). In the figures seen, the chromatin was oriented at metaphase between two poles of a spindle; but because of the small size of the nucleus, I have been unable to ascertain the number of chromosomes present at this stage. Around the spindle there is an area of more lightly staining plasma the size of the original nucleus, which may indicate that the spindle is intranuclear.

In *Tuomeya* free spermatia found in apparent nuclear division seem to indicate a division occurs before attachment of the spermatia to the trichogyne. When attached to the latter, the spermatium possesses two darkly staining granules (Plate 3, Figs. 24, 25) which I believe are nucleoli of two daughter nuclei resulting from the division. In a few cases I have observed a clear zone around each of these granules, similar to the clear plasma of the interphase nucleus, and the size of the nuclei appears to be approximately the same as that of male nuclei seen within the trichogyne.

THE CARPOGONIAL BRANCH

The carpogonium is borne at the tip of a special branch which arises from the basal cell of a lateral assimilative branch (Plate 3, Fig. 26; Plate 4, Figs. 27, 28). There may be one or two carpogonial branches borne at each node: they grow upward into the hollow space between the cells of lateral branches and the main axis.

The carpogonial branch is a spirally twisted structure composed of usually ten to fourteen somewhat egg-shaped cells with the larger end of each toward the convex side of the spiral (Plate 3, Fig. 26). The twisting of the branch is quite characteristic. Occasionally, the cells of the proximal half of the branch produce short filaments of cylindrical cells which bear assimilative cells at their tips and grow out toward the periphery of the frond (Plate 3, Fig. 26). Each cell of the distal half of the branch produces a row of one to four short, rounded cells which function as nutritive cells for the gonimoblast (Plate 3, Fig. 26; Plate 4, Figs. 27, 28), and which are comparable to those "nutritive cells" on the carpogonial branch of the *Nemalionales,* which are described by Kylin (1935) as food suppliers for the development of the gonimoblast.

The cells of this branch possess a central vacuole, and when young there is a well developed chromatophore. At maturity those cells of the proximal half retain their chromatophores, but those of the distal half break down into a dense granular plasma (Plate 4, Figs. 27, 28). Each cell possesses a single nucleus similar in structure to those already described for the vegetative cell.

The young carpogonium is at first elongated and somewhat constricted near the tip but is still not differentiated into the three portions of the mature one (Plate 4, Fig. 29). There is in the young carpogonium a parietal chromataphore which soon breaks down, leaving only a small chromatophore rudiment at the base (Plate 4, Fig. 27). This globular body takes a dull gray stain with iron hematoxylin. In *Tuomeya* there was no evidence of a chromatophore stretching up into the trichogyne from the carpogonium. A single nucleus is present near the base of the cell in the young carpogonium. At a later stage, but before fertilization, a division apparently takes place, for the carpogonium appears to be bi-nucleate (Plate 4, Figs. 28, 30). One nucleus occurs in the carpogonium and the other in the trichogyne. The trichogyne nucleus has a small nucleolus and around it a lightly staining zone which is sometimes hard to differentiate from the cytoplasm. The trichogyne, at maturity, becomes so granular that it is difficult to say exactly what becomes of the nucleus; but just prior to fertilization it is not apparent and may be in a state of degeneration.

At the time for fertilization the procarp of *Tuomeya* consists of a swollen basal portion, the carpogonium, in which lies the egg nucleus, a larger somewhat elongated, pear-shaped trichogyne at the distal end, and a narrow, tu-

bular portion called the pedicel connecting the two (Plate 4, Fig. 27). The granular protoplasm is continuous through the pedicel between the carpogonium and trichogyne.

FERTILIZATION

The non-motile spermatia, after being discharged from the spermatangia, are carried to the trichogyne by currents of water. Often as many as five or six spermatia may be attached to one trichogyne.

The spermatium, as we have seen, is bi-nucleate when it comes in contact with the trichogyne (Plate 3, Figs. 24, 25), and after the intervening walls have dissolved, the nuclei pass into the trichogyne (Plate 3, Fig. 24; Plate 4, Fig. 31). Sometimes a small amount of cytoplasm is left within the old spermatium wall (Plate 4, Fig. 32). One of the two nuclei passes down through the pedicel into the carpogonium while the other remains in the trichogyne. Soon after, the protoplasmic connection between the carpogonium and the trichogyne is broken by a thickening of the wall of the pedicel (Plate 3, Fig. 24; Plate 4, Figs. 32, 33).

Fusion of the male and female nuclei appears to take place soon after the male nucleus enters into the carpogonium (Plate 3, Fig. 24; Plate 4, Fig. 32). In a single frond, various stages occur from fertilized carpogonia near the upper end to carpospore-forming stages below, and only a few stages show fusion. In the carpogonium both nuclei appear to be in an interphase condition.

After fertilization the carpogonium bulges out at one side, and a longitudinal wall divides it into two cells, each of which possesses a single nucleus (Plate 3, Fig. 25; Plate 4, Figs. 33, 34). These two cells then divide, thus forming a quartet of cells, which form a row at the tip of the carpogonial branch.

The gonimoblasts in *Tuomeya*, which develop from the four cells described above consist of two parts, a primary and secondary gonimoblast (Plate 4, Fig. 35; Plate 5, Fig. 37). The former are composed of short chains of more or less polygonal cells (Plate 4, Figs. 35, 36; Plate 5, Figs. 37, 38, 39), the outermost of which dichotomize at the distal end and thus form a cluster of cells around the old carpogonium. The secondary gonimoblast is composed of long filaments of cylindrical cells. Two such filaments may arise from each of the end cells of the primary gonimoblasts and grow out toward the periphery of the frond and there give rise to the carpospores (Plate 5, Fig. 37).

In *Tuomeya* the cytoplasm of cells of the distal half of the carpogonial branch and the primary gonimoblast is densely granular and of similar consistency (Plate 4, Fig. 35; Plate 5, Figs. 38, 39, 40). Later the primary

gonimoblast cells fuse with the quartet of cells formed from the division of the fertilized carpogonium and also with cells of the distal end of the branch and their nutritive cells. Thus, a large fusion cell or gonimoblast placenta is formed which gives rise to secondary gonimoblast filaments (Plate 4, Figs. 35, 36; Plate 5, Figs. 37, 38, 39, 40, 41). The fusion of cells into this gonimoblast placenta is so complete that it is difficult to tell one cell from another. The placenta is densely granular and usually contains numerous large nuclei which are budded off as nuclei of the primary gonimoblast cells. Occasionally I have found these nuclei undergoing mitotic division (Plate 5, Figs. 39, 40).

At metaphase of the divisions seen in the nuclei of the gonimoblast placenta, the chromatin occurs in about eight or nine small chromosomes. This would seem to indicate that a reduction division has taken place, for as nearly as can be estimated, it corresponds with the number of granules in the spermatium. The number of granules seen in the prophase stage of the spermatium I have considered indicative of the haploid chromosome number.

Although reduction division has not been observed in *Tuomeya*, it is likely that it occurs at the first division of the zygote as it does in other haplobiontic red algae. Two observations seem to indicate that this may be true: first the development of the fertilized carpogonium into four gonimoblast initials; second, the presence of the same number of chromosomes in the dividing nuclei of the gonimoblast as were seen in the discharged spermatium.

Cells of the secondary gonimoblast possess a parietal chromatophore that is irregularly thread-like with frequent thickenings of the thread (Plate 4, Fig. 35; Plate 5, Fig. 39). As a rule these filaments do not branch until they are near the periphery of the frond, where they usually dichotomize once or twice (Plate 5, Figs. 37, 40, 41) and at the surface of the frond bud off successive carposporangia (Plate 5, Figs. 41, 42). The latter are terminal cells of these filaments.

Each carposporangium produces a single carpospore which may be somewhat cylindrical when first formed but becomes a swollen egg-shaped structure when mature (Plate 5, Fig. 41). The carposporangium is densely filled with protoplasm and the chromatophore is prominent. As the spore increases in size, the chromatophore diminishes until ultimately it does not show as such, but only as small globules of pigment. The carpospore cytoplasm appears reticulate and granular. The nucleus, as a rule, occupies the central portion of the cell. The discharge of the spore occurs by rupture of the distal end of the carposporangium wall (Plate 5, Figs. 41, 42); and, after it is freed, a new carposporangium may arise within the wall of the preceding one.

The carpospore rounds up after being discharged and soon may germinate to form the juvenile plant described previously.

DISCUSSION

In 1858 *Tuomeya fluviatilis* was named and placed in the sub-class Chlorospermae, now known as the Chlorophyceae, by Harvey. He believed that *Batrachospermum* and *Lemanea* were "inseparately connected by the genus *Tuomeya*." Setchell (1890), from his study of *Tuomeya* came to the conclusion that this genus was an intermediate one between *Batrachospermum* and *Lemanea*. In subsequent classifications, *Tuomeya* has been placed in the Lemaneaceae. Kylin (1937) left *Tuomeya* in the Lemaneaceae, but stated that it might equally well be placed in the Batrachospermaceae. From my study of *Tuomeya* however, I am inclined to regard this genus as a member of the Batrachospermaceae.

As in *Batrachospermum moniliforme* (Kylin, 1917) the spermatangium mother-cell of *Tuomeya* cannot be distinguished from an ordinary assimilatory cell, whereas in *Lemanea* it is distinctly different. The spermatia of *Lemanea* are developed in special areas or sori. The presence of a chromatophore in the spermatium of *Tuomeya* also resembles the development of a similar parietal chromatophore in the spermatium of *Batrachospermum* as reported by Davis (1896), Schmidle (1899), Osterhout (1900), and Kylin (1917).

The granules appearing in the nucleus of the spermatangial cell as observed in *Tuomeya* are not uncommon among the Florideae. In *Batrachospermum* and *Nemalion* these granules were thought by Kylin (1916, 1917) to indicate a prophase stage as I believe to be true in *Tuomeya:* and in many of the Florideae investigated it has been established that the nucleus is in a prophase stage when the spermatium is liberated.

A mitotic division of the nucleus of the spermatium as observed in this study of *Tuomeya* has also been reported in *Nemalion* (Kylin, 1916; Cleland, 1919) and *Batrachospermum* (Schmidle, 1899). Spermatia attached to the trichogyne of *Batrachospermum* were observed by Kylin (1917) to possess two darkly staining granules which he said may indicate a binucleate condition. He pointed out that the spermatium when it is first discharged is in a prophase stage, and later, when it lies in the carpogonium prior to the fusion with the egg nucleus, it is in the interphase condition. He believed this to be evidence that a nuclear division had taken place and regarded the observation of Schmidle as correct.

It is likely that the only spermatia Setchell (1890) saw in *Tuomeya* were those attached to the trichogyne, for he undoubtedly confused spore-bearing gonimoblast filaments for spermatangial branches; the carpospores, produced at the ends of long secondary gonimoblast filaments he confused with spermatia or antheridia. His description of the spermatium fits the carpospore quite accurately. He did not describe the carpospore but did state that "as far as could be seen, strings of spores similar to those formed in *Batracho-*

spermum seemed to be produced." The carpospores of *Tuomeya* however, are not produced in "strings" but are borne singly at the ends of long cylindrical gonimoblast filaments.

A chromatophore which stretches up into the trichogyne from the carpogonium has been reported present in *Batrachospermum* by Davis (1896), Schmidle (1899), Osterhout (1900), and Kylin (1917). I was never able to see such a chromatophore in *Tuomeya* nor was one reported by Setchell in his study of this plant.

Among the Florideae which have been studied according to Kylin (1917), it appears as if the trichogyne normally contains a nucleus. The presence of a trichogyne nucleus in *Batrachospermum* was reported by Davis but Schmidle, Osterhout and Kylin were unable to demonstrate such a nucleus. In *Nemalion* Kylin (1916) said there is a small trichogyne nucleus present which is seen only with difficulty. In older trichogynes he said the nucleus usually disappears. Kylin (1923) also reported a nucleus present in the trichogyne of *Lemanea fluviatilis.* In *Tuomeya* the trichogyne nucleus disappears but is present up to about the time of fertilization; so in this respect *Tuomeya* apparently does not resemble *Batrachospermum* or perhaps the observation of Davis is the correct one, since according to Kylin, among the Florideae studied, the trichogyne normally possesses a nucleus.

The spermatium and egg nuclei in *Tuomeya* appear to be in the interphase stage of mitosis at the time of fertilization as is true of *Batrachospermum* (Kylin, 1917) and *Nemalion* (Wille, 1894; Kylin, 1916; Cleland, 1919). In such forms as *Polysiphonia violacea* (Yamanouchi, 1906), *Griffithsia corallina* (Kylin, 1916a.) and *Scinaia furcellata* (Svedelius, 1915) the male nucleus is in a prophase stage when discharged; it does not divide but is said to pass down the trichogyne in a prophase stage.

After fertilization in *Tuomeya* and after the probable meiotic division of the zygote nucleus has taken place forming the quartet of cells, as reported earlier in this paper, the gonimoblast begins to develop. This type of development was first observel by J. G. Agardh (1863) in *Asparagopsis Delilei* Mont., for he pointed out a distinct difference between the first cells produced by the gonimoblast and the outer cells which bear carpospores at their tips. Two types of gonimoblast cells are reported for *Gracilaria* by Kylin (1930) and for *Asparagopsis armata* by Svedelius (1915). Svedelius said it is not unusual but certainly not common for the gonimoblasts in the Rhodophyta to differentiate into a central part and another spore-bearing part. *Tuomeya* and *Batrachospermum* are not similar in this respect, for this type of development has never been reported for the latter.

The development of a gonimoblast placenta, by the fusion of the primary gonimoblast cells with the four gonimoblast initials at the distal end of the carpogonial branch and their nutritive cells as observed in *Tuomeya,* does not occur in *Batrachospermum* according to Kylin (1917). In the latter, the

gonimoblast is nourished through the cells of the carpogonial branch, but these do not fuse with each other. However, the fusion of sterile cells with the gonimoblast is reported in *Nemalion* by Kylin (1916). In *Bonnemaisonia asparagoides*, Svedelius (1933) said, there is a fusion of the carpogonium with the hypogynous cells and the nutritive cells borne on it. These cells furnish only part of the food of the gonimoblast. The greater part of the food is obtained from other cells of the mother plant and is not conducted to the gonimoblast through the carpogonial branch. In *Asparagopsis armata* Svedelius said the cells of the carpogonial branch fuse with one another and their content is used as food for the development of the gonimoblast; here though, the food from all cells is transported to the gonimoblast through the carpogonial branch.

It has been noted previously concerning the nucleus of the spermatium of *Tuomeya* that a number of granules which were thought to be chromosomes appear. The nucleus was thought to be haploid and in a prophase stage. The number of granules in the spermatium nucleus seems to agree with the number seen in the gonimoblast nuclei undergoing mitosis. For this reason it would appear that reduction division had taken place; about 7 to 9 granules or chromosomes occurred in the spermatium nucleus and in the gonimoblast, a structure which follows fertilization. In *Scinaia furcellata* (Svedelius, 1915), *Asparagopsis armata* and *Bonnemaisonia asparagoides* (Svedelius, 1933), the number of granules which was seen in a prophase stage of the spermatium nucleus was considered to be the haploid number. Kylin has said that in the case of *Nemalion multifidum* and *Batrachospermum moniliforme*, the granules of the mature spermatium nucleus correspond with the haploid number. Yamanouchi (1906) refers to granules occurring in the spermatium of *Polysiphonia violacea* as prochromosomes that are connected by weakly staining threads of linin. These prochromosomes he said, increase in size and become rod-shaped chromosomes. Kylin (1916) stated that the characteristic granules in the mature spermatium nucleus are observed in all Florideae. Thus, although reduction division was not observed in *Tuomeya* it would seem likely that it occurs at the first division of the zygote nucleus, and Kylin (1937) states that such is the case in other haplobiontic red algae. According to Kylin (1917), if the first division of the zygote in *Batrachospermum* is heterotypic, the second would be homotypic, and the four cells resulting from these two divisions would be homologous with the tetraspores which arise after reduction division in the higher red algae. Two observations seem to indicate that this may be true in *Tuomeya fluviatilis;* first, the development of the fertilized egg into four gonimoblast initials which are similar to those found in *Batrachospermum* where meiosis occurs in the zygote nucleus; second, the presence of what is considered to be the haploid number of chromosomes in dividing nuclei in the gonimoblast.

The carpospore formed singly in the terminal cells of the secondary

gonimoblasts of *Tuomeya* is unlike the carpospore development in *Lemanea*. Smith (1950) partly characterizes the family Lemaneaceae by the fact that "carpospores are formed by all the cells of the gonimoblast instead of from the terminal cells only." Of the Batrachospermaceae he says, "the gonimoblasts formed from the carpogonia develop carpospores from their terminal cells only."

When the aspect, general morphology, and cytology are considered, it is evident that *Tuomeya* is out of place in the family Lemaneaceae and should be placed in the Batrachospermaceae.

SUMMARY

1. Carpospores of *Tuomeya* germinate to form a juvenile plant on which monospores are formed.
2. Basal filaments of the juvenile plants give rise to the mature plants.
3. Spermatia are borne at the tips of the lateral assimilative branches and when discharged from the spermatangia undergo mitosis.
4. When first attached to the trichogyne, the spermatia are bi-nucleate.
5. The unfertilized carpogonium is also bi-nucleate; one nucleus lies at the base of the carpogonium while the other is in the trichogyne.
6. Fusion of the male and female nuclei occurs soon after the male nucleus enters into the carpogonium.
7. The fertilized egg cell divides to form four cells from which gonimoblasts, with two types of cells are developed.
8. Fusion of numerous cells takes place to provide a gonimoblast placenta which gives rise to primary and secondary gonimoblasts.
9. Carpospores are produced singly at the tips of secondary gonimoblast filaments at the edge of the frond.
10. Mitotic divisions in the gonimoblast placenta show about eight chromosomes; the same number was thought to be present in the haploid spermatium.
11. Reduction division is thought to occur at the first division of the fertilized egg cell.
12. It is suggested that *Tuomeya fluviatilis* Harv. be placed in the family Batrachospermaceae rather than the Lemaneaceae where it now stands.

EXPLANATION OF PLATES

Lettering of figures: ac, assimilative cell; cac, central axis cell; cf, corticating filament; ch, chromatophore; cpg, carpogonium; cpgb, carpogonial branch; cspm, carposporangium; gi, gonimoblast initial; lbi, lateral branch initial; mspm, monosporangium; nc, nutritive cell; p, pedicel of carpogonium; pgf, primary gonimoblast filament; sgf, secondary gonimoblast filament; sp, spermatium; spm, spermatangium; spmc, spermatangium mother-cell; tr, trichogyne; tn, trichogyne nucleus; ♂ N, male nucleus; ♀ N, female nucleus.

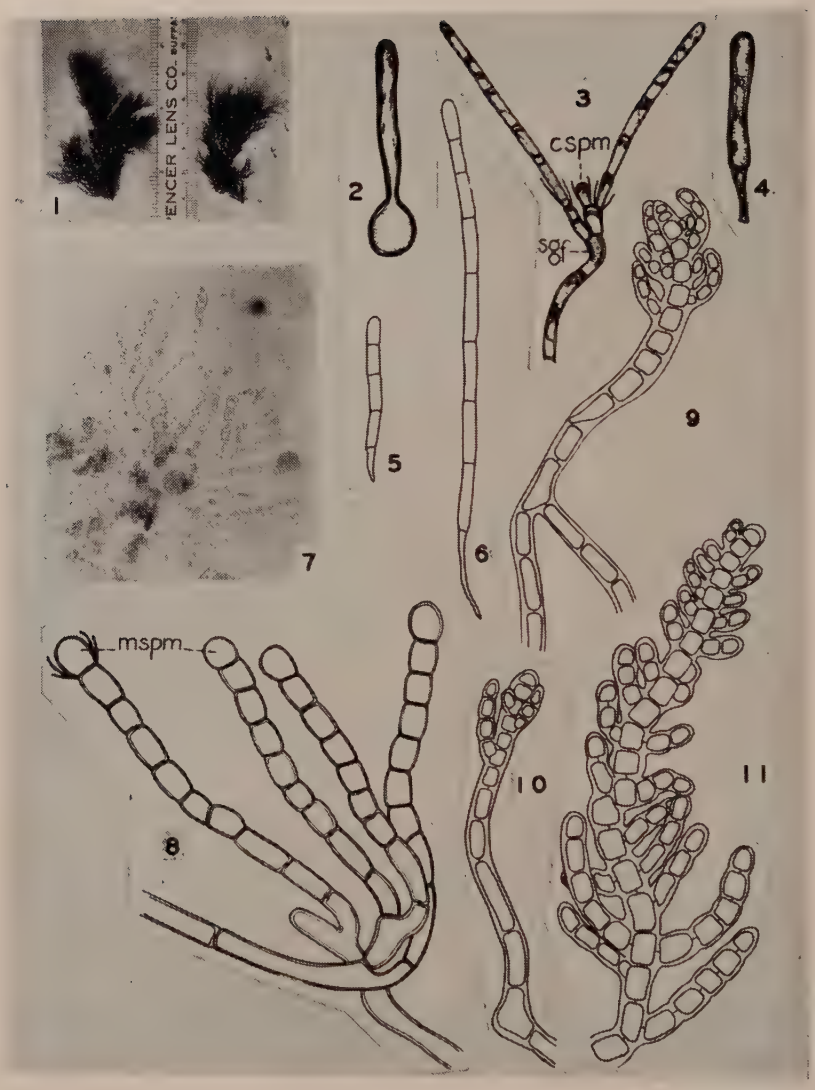

PLATE 1

Fig. 1. Photograph of mature plants of *Tuomeya*. ½ natural size. Fig. 2. Germinating carpospore. X892. Fig. 3. Carpospore germination on parent plant. X625. Fig. 4. Young germling after spore wall has fallen off and first cross-wall has formed. X892. Fig. 5. Young sporeling, five cells long. X625. Fig. 6. Later stage showing sporeling of nine cells. X625. Fig. 7. Photomicrograph of juvenile plant showing the basal mat of interwoven branched filaments and the erect filaments of moniliform cells. Monospores at the tips of the erect filaments may be seen. Fig. 8. Outline drawing of juvenile plant with terminal monosporangia. X892. Figs. 9-11. Stages in the development of sexual frond from cells of the basal mat of the juvenile plant. X625.

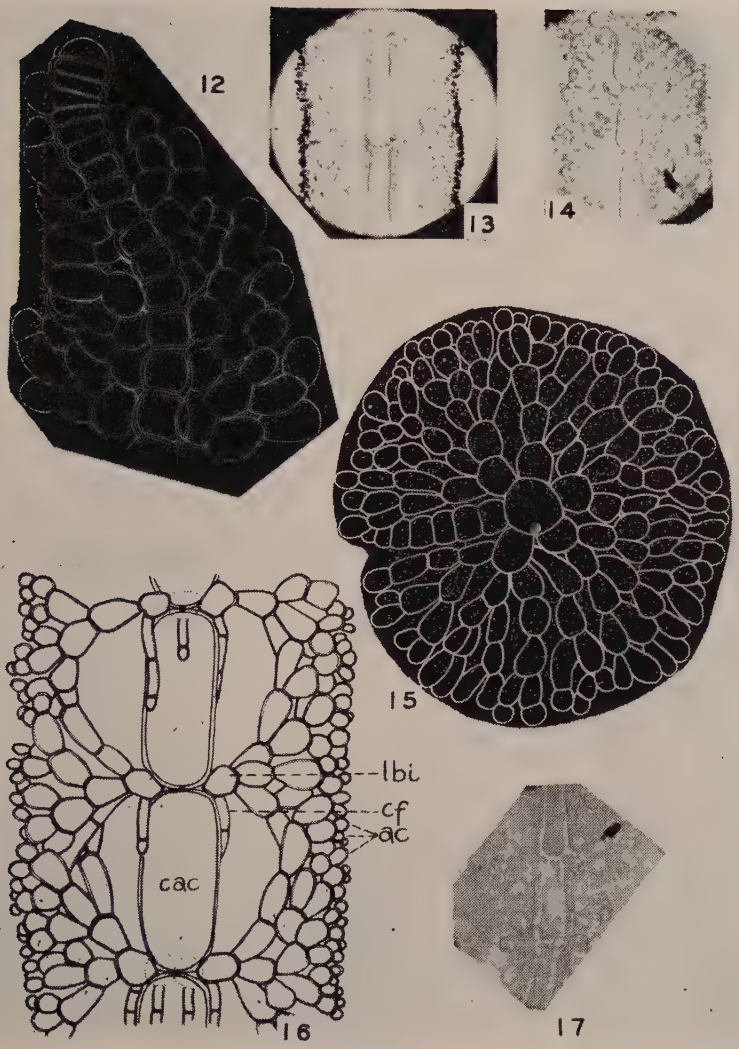

PLATE 2

Fig. 12. Apical region of sexual frond, showing apical cell; young axial cells cut off from its base have given rise to lateral branches. X1000. Fig. 13. Photomicrograph of longitudinal section of a mature frond, showing elongated central axis cells, corticating filaments, and lateral branch origin and development. Fig. 14. Photomicrograph of a younger portion of frond crushed out on a slide showing the differentiation of axis cells at base and apex. Fig. 15. Cross section through a node in young portion of frond, showing origin of lateral assimilative branches and main axis branch. X892. Fig. 16. Longitudinal section of a young frond showing the elongating central axis cells, lateral branch system and corticating filaments. X892. Fig. 17. Photomicrograph of crushed material showing the origin of corticating filaments from base of lateral branch initial.

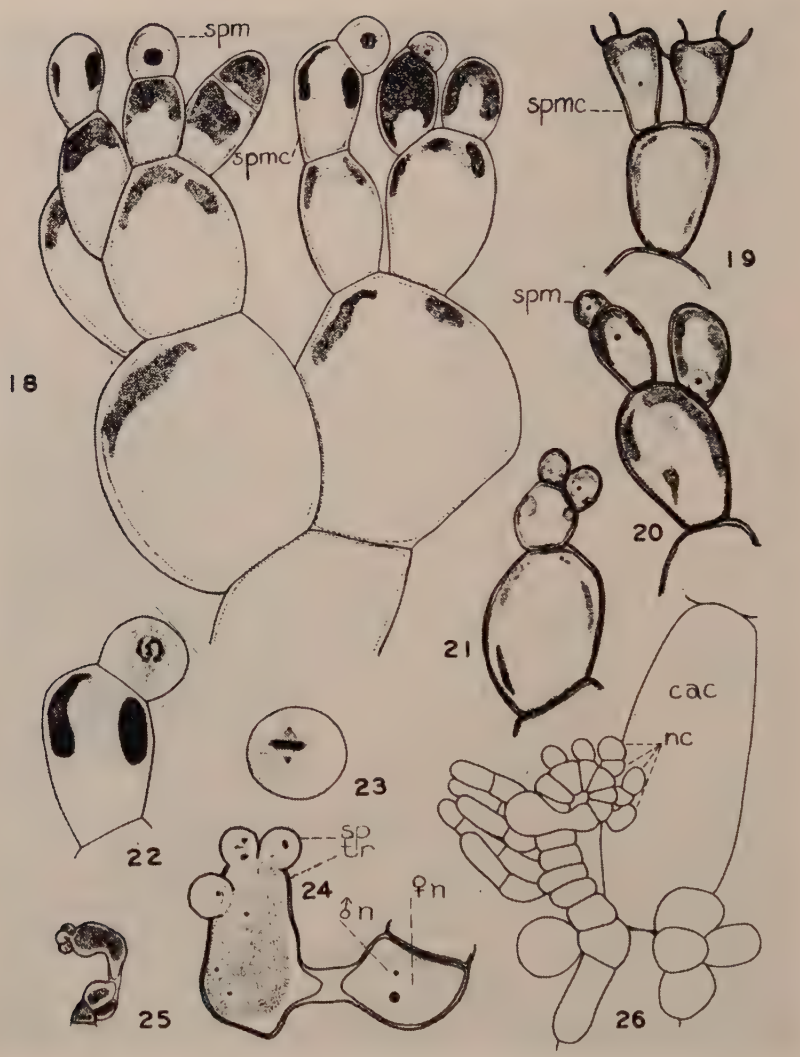

PLATE 3

Fig. 18. Portion of a plant showing spermatangia mother-cells and spermatangia. The nuclei of two spermatangia are in the prophase stage. X2000. Fig. 19. Spermatangia mother-cells after they have become heart-shaped from production of spermatangia. Spermatia have been discharged. X1785. Figs. 20, 21. Spermatangia showing parietal chromatophores. X1785. Fig. 22. Portion of Fig. 18 enlarged, showing spermatangium nucleus in prophase stage with eight granules visible. X3000. Fig. 23. Spermatium after discharge from the spermatangium showing nuclear division at metaphase. X3000. Fig. 24. Spermatia attached to trichogyne showing binucleate condition in one. Two other spermatia visible, each with a single nucleus. Three male nuclei occur in the trichogyne and one is in the process of fusing with the egg nucleus. The fourth spermatium is not visible. X2000. Fig. 25. Two spermatia in binucleate condition, attached to trichogyne; the first division of the fusion nucleus has taken place. X892. Fig. 26. Outline drawing from an aceto-carmine smear showing carpogonial branch and its relation to the central axis. X1000.

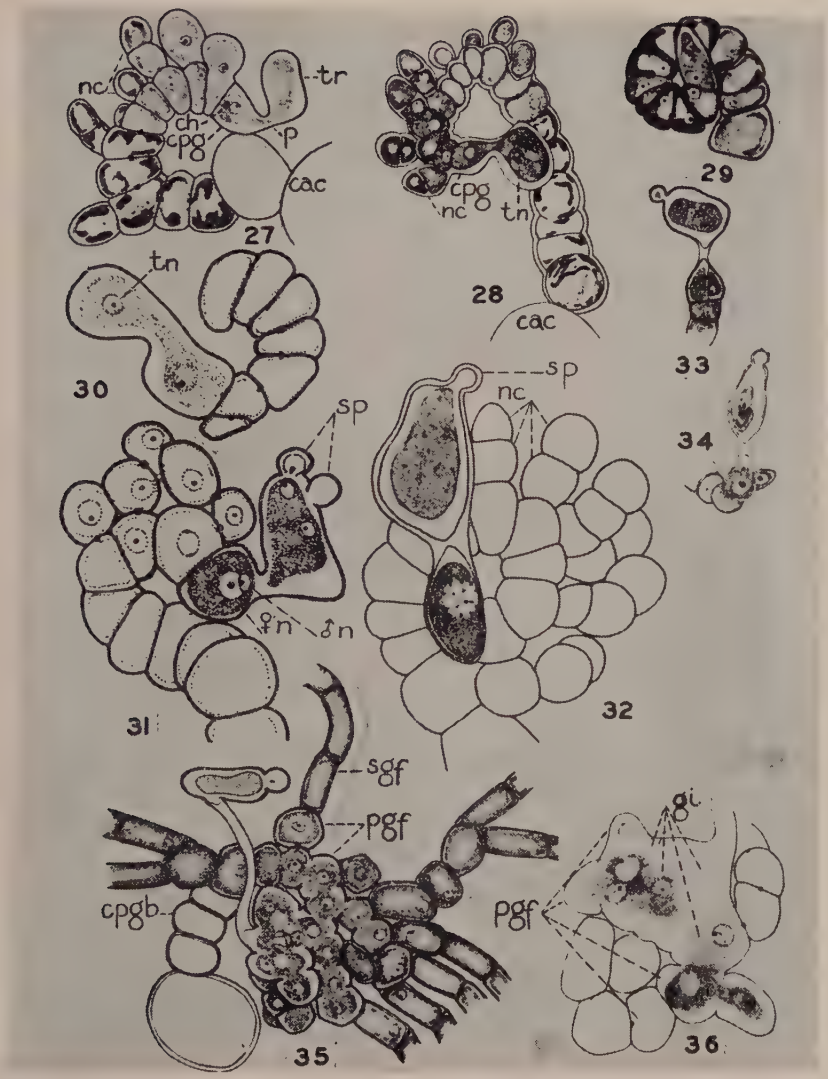

PLATE 4

Fig. 27. Carpogonial branch showing chromatophore rudiment in the carpogonium and degeneration of chromatophores of distal end of branch. X1000. Fig. 28. Same structure showing nucleus in trichogyne. X1000. Fig. 29. Young carpogonial branch before elongation of trichogyne and growth of nutritive cells. X1000. Fig. 30. Portion of a carpogonial branch showing binucleate carpogone. X2000. Fig. 31. Carpogonium after fertilization showing male and female nuclei fusing. X2000. Fig. 32. Carpogonium after fusion of male and female nuclei. About 14 granules were evident. X2000. Fig. 33. First division of the carpogonium after fertilization. X892. Fig. 34. First division of the carpogonium after fertilization. X892. Fig. 35. Carpogonial branch showing fusion of four gonimoblast initials and development of primary and secondary gonimoblast cells. X1000. Fig. 36. Portion of branch at an earlier stage showing fusion of gonimoblast initials and first primary gonimoblast cells being formed. X1000.

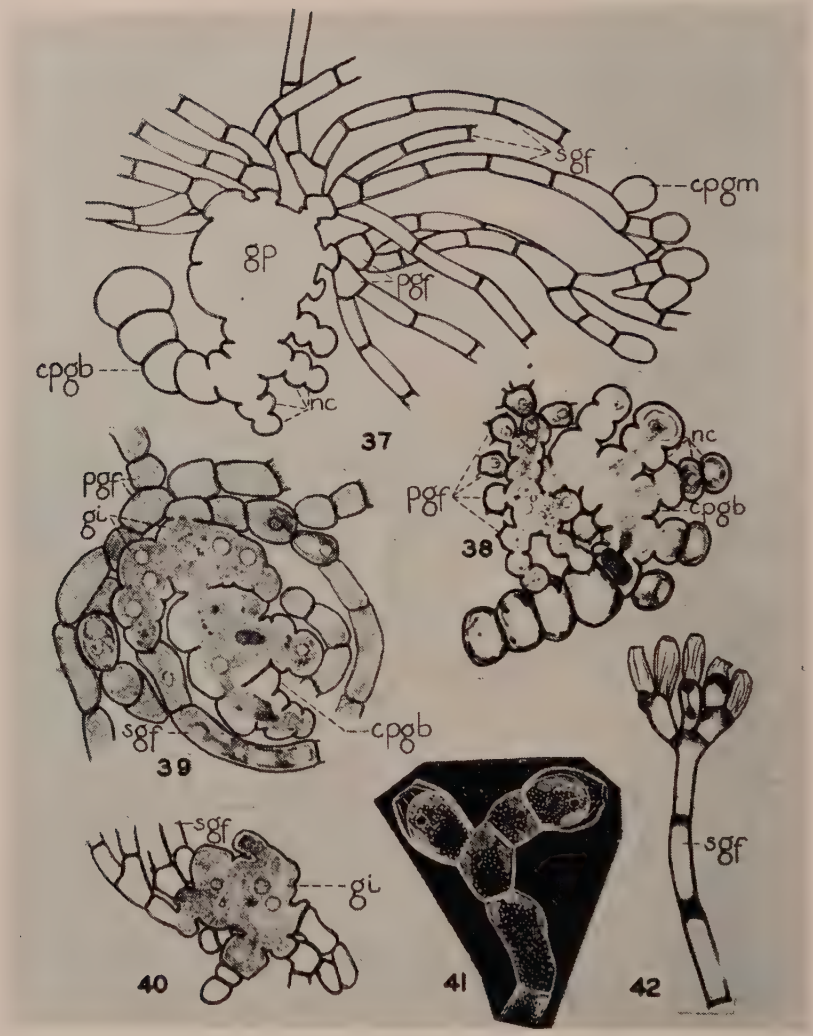

PLATE 5

Fig. 37. Outline drawing showing fusion cell or gonimoblast placenta with secondary gonimoblast filaments bearing carpospores. X1000. Fig. 38. Carpogonial branch showing fusion of branch and nutritive cells with gonimoblast initials and primary gonimoblasts. X1000. Fig. 39. Later stage showing fusion. Secondary gonimoblast filaments may be seen arising from the primary cells. X1000. Fig. 40. Gonimoblast placenta with one nucleus undergoing nuclear division. Eight granules may be seen in polar view. X1000. Fig. 41. Secondary gonimoblast filament bearing two carposporangia within the walls of two previous carposporangia. X2000. Fig. 42. The same, showing branching of filament and walls of old carposporangia. X1000.

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A SIMPLIFIED MEDIUM FOR USE IN TISSUE CULTIVATION OF POLIOMYELITIS VIRUS

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Dr. John Enders' (1) outstanding discovery in 1949 that all three types of Poliomyelitis virus would grow in tissue culture of extraneural cells, has paved the way for Dr. Jonas Salk's success in developing a safe and effective Poliomyelitis virus vaccine. Although virus vaccines are not new, this vaccine developed by Dr. Salk is the first to be produced on a large scale in tissue culture for human use. The advances made against this dreaded disease were made possible by the improvements in tissue culture techniques. One of the more important advances has been the development of a synthetic medium known as Medium 199 (2). Although this medium has proved satisfactory in supporting the growth of tissue for the cultivation of Poliomyelitis virus, the expense and the limited supply of the large number of amino acids, nucleic acids, and vitamins of which the medium is composed, and the complexity of its preparation has made this medium costly and tedious to produce.

This study was undertaken with the hope of finding a simpler medium for the tissue cultivation of Poliomyelitis virus for use in vaccines.

REVIEW OF LITERATURE

The advance made in Poliomyelitis research has coincided with the development of tissue culture media and improvements in tissue culture techniques. Since the classical observation of the *in vito* growth of embryonic neural tissue by R. J. Harrison (3), volumes of work have accumulated on procedures that permit the survival or growth of cells outside the body. A variety of media have been used for maintenance and growth of tissues. The monograph of Parker (4) gives an extensive description of techniques used in tissue culture as well as media that allow for the growth of cells.

The media employed for the growth of tissue cultures prior to 1950 have been derived directly or indirectly from the organism and consist of blood plasma, blood serum, embryo tissue extract, body exudates, and extracts of various tissues and organs mixed with a physiological salt solution. Though such media are unsatisfactory for cultivating virus for vaccine purposes and for studying the nutritional requirements of the cell, the studies on the nutrition of the cell are worth mentioning for the information these papers give on substances found to have cell growth stimulating properties.

Lewis and Lewis (5) were among the first to study the behavior of cells

in media of known composition. In 1911, they investigated the survival of chick embryo tissues in solutions containing varying concentrations of sodium chloride, calcium chloride, potassium chloride and sodium bicarbonate, and found that growth was influenced to a marked degree by variations in the proportion of these salts. They also found that excellent growth could be obtained in solutions of amino acids and polypeptids of known composition and that media containing dextrose or maltose were better than pure salt solutions. Unfortunately, no precise details of these media were supplied. Later, work by Burrows and Neyman (6) indicated that hydrolytic products of egg yolk, and mixtures of ten amino acids were toxic for chick embryo cells. From this work the concept that amino acids are toxic for tissue cultures has persisted until recent times.

Carrel (7) in 1913, focused attention upon the role of proteins in cell nutritions by noting that extracts of tissues and particularly embryo tissues greatly accelerated the growth of fibroblasts. Carrel and Baker (8) in 1926, discovered that fibroblasts, epithelial cells, and monocytes, when cultivated in plasma, obtain their nitrogen from proteoses, and possibly other split products of the protein. They also found that peptic digests of egg white and fibrin which contained large amounts of proteoses and peptones, stimulated the cells to greater activity than did tryptic digests which consisted almost entirely of the lower, less complex, hydrolytic products of proteins. They concluded that the cells do not feed on amino acids and other ultrafilterable constituents of embryo extracts, but rely on proteins or closely related fractions. Commercial peptones gave variable results, although Witts' peptones appeared suitable. In 1928, Baker and Carrel (9) fractionated Witts' peptone and showed that alpha and beta proteoses were equal as growth promoting substances and they compared the liver hydrolysates of pepsin, trypsin, and hydrochloric acid. They noted that the products of the peptic digest were more stimulating to growth of the cells than were the other digests prepared with trypsin and hydrochloric acid. They also showed that amino acids contribute to the growth of cells, but are unable to maintain the viability of the cell without the addition of peptids or polypeptids to the medium. In another report of the same year Baker and Carrel (10) further established that the pepsin hydrolytic products of crystalline egg albumin, purified casein, crystalline edestin and purified fibrin are utilized by fibroblasts for their proliferation. It might be noted that the above products were used in combination and no success was observed when any of the hydrolytic products were used alone. The results of Baker and Carrel warrant their conclusion that protein degradation products alone do not provide an adequate diet for all types of cells; the presence of plasma, embryo extract or serum is also necessary. This supplementary action of complex natural substances has been attributed to the presence of enzymes that slowly digest the proteoses to utilizable amino acids.

In 1936, Baker (11) developed a "feeding solution" for fibroblasts and epithelial cells which is noteworthy in that he recognized the importance of a vitamin supplement. Vitamin A, ascorbic acid, Vitamin D, glutathione, and 10 per cent serum were shown to be beneficial for growth of fibroblasts and epithelial cells. The addition of a Vitamin B complex aided in the cultivation of Monocytes. In 1939, Baker and Ebeling (12) attempted to devise a fluid that would maintain cells without promoting growth. The fluid adopted was similar to the "feeding solution" of 1936, with the addition of ten other substances which included two hormones. Cultured strains of fibroblasts were maintained in a viable condition and with little or no proliferation for periods from forty-three to fifty-six days.

Fischer and his associates (13) published a monograph in 1946, summarizing their work of twenty years on cell nutrition. Fractionation studies on embryo tissue juice revealed that the growth promoting activity was associated with a labile substance of high molecular weight. It was found to occur in the nucleoprotein fraction and was similar to the fraction reported by Baker and Carrel. Fischer believed that the mechanism of action of this substance on cells was a catalytic one. Dialysis of plasma, serum, or embryo extract against ringer-glucose solution resulted in the loss of low molecular weight substances and such substances were found essential for the metabolism of the cells. On the supposition that dialyzed media would be deficient in free amino acids, he investigated the ability of different amino acid solutions to compensate for the substances lost by dialysis. In these studies, Fischer observed that cystine, lysine, and glutamic acid were indispensable for the metabolism of the cells.

Astrup, Fischer, and Volhert (14) studied the distribution of substances in animal and vegetable tissues that could be substituted for the dialyzable constituents of serum or embryo juice and found that active growth stimulants could be obtained from kidney, yeasts, barley malts, and calf embryo muscle.

Fischer and his associates (15, 16) using all substances known to be of biological importance developed two mixtures to which were added dialized plasma, embryo extract, and certain inorganic salts. By deletion of individual amino acids, he attempted to determine which were essential for growth of in vitro cells. Fischer arranged the amino acids in the order of their decreasing importance in growth as cystine, arginine, tryptophane, glutamine, and lysine, and also employed crystalline trypsin and pepsin to prepare digests of pure lactoglobulin and bovine serum albumin. He then devised mixtures of pure amino acids in the proportions found in these proteins and compared the cell growth that was obtained. Invariably the protein digests caused a much greater growth response than did the amino acid mixtures. Fischer concluded that tissue cultures may require an unidentified growth promoting factor of peptid or polypeptid nature.

In 1946, a synthetic medium was devised by White (17) consisting entirely of constituents of known composition in Locke's Solution in which chick embryo tissues were grown in roller tubes directly on the surface of the glass. By this method complex materials such as plasma, serum, and embryo extract were eliminated and the effect of various mixtures of chemically known growth substances were tested directly. White reported this medium maintained skeletal fibroblast of eight day chick embryos in good condition for fifty-eight days and kept heart muscle beating for forty-four days. White's Medium did not contain glutamic acid or cystine, which were the two amino acids found by Fischer to be so important in cell nutrition.

In 1950, Morgan, Norton, and Parker (2) reported on a synthetic medium called Medium 199. Initially a basal solution of casein hydrolysate in Earl's Salt Solution was tried, but proved to be unsuccessful and was replaced with an amino acid basal solution. By a gradual building up process these investigators arrived at Medium 199. This medium was composed of 20 different amino acids, 17 vitamins, 2 lipids, 10 nucleic acid derivatives, phenol red indicator, glucose and a number of inorganic salts. Tissues from the leg muscles of eleven day old chick embryos were cultivated directly on the surface of the glass in roller tubes. In contrast to White, these investigators reduced the amount of tissue in the roller tubes to a minimum in order to eliminate as far as possible the carryover of nutrient substances from the embryo. They reported cells proliferated and cultures remained in good condition for an average period of four to five weeks.

Although no wholly adequate synthetic medium for tissue culture has been devised, this medium of Morgan, Norton, and Parker represents the most complete one devised up to this time. Medium 199 has proved satisfactory for the tissue cultivation of Poliomyelitis virus (18, 19, 20).

Morgan, Morton, and Parker (2), employing Medium 199 as a basal solution, studied the effect of the supplementary addition of serum and embryo extract. They observed that the addition of serum from 0.1 to 20 per cent, did not significantly improve the growth promoting qualities of the synthetic mixture, but the addition of embryo extract, at concentrations of 0.25 to 5.0 per cent, proved highly beneficial and resulted in culture growths comparable to those obtained in media comprised of serum and embryo extract. It was concluded that some factor or factors present in embryo extract were still missing from this synthetic medium.

In 1953, Evans, Shannon, Earl, and Sanford (21) attempted to ascertain whether either White's or Medium 199 could be used as a basal nutritional medium to maintain suspended mammalian cells (mouse fibroblastic cells). These two media were compared with the following: (1) EBS (Earl's Balanced Salt Solution), (2) EBS and horse serum, and (3) EBS, horse serum, and chick embryo extract. Their data indicated that White's Medium and

Medium 199 were not adequate basal media since neither could maintain mammalian cells.

In another series of experiments, Sanford, Evans, and Earl (22) demonstrated that the protein fraction of chick embryo extract and the ultrafilterable non-protein fraction of horse serum can be omitted from the serum-extract medium without reducing the cell proliferation if the remaining two fractions are supplemented by certain amino acids. Using Medium 199 and different portions of this medium as supplements, it was demonstrated that the amino acids and possibly the vitamin portions were the essential materials in Medium 199.

In 1952, Melnick and Riordan (23) found that the protein containing substances of the serum-embryo extract culture medium could be replaced by a lactalbumin hydrolysate. When lactalbumin hydrolysate is used instead of embryo extracts in nutrient medium, fibroblasts from monkey testicular tissue plasma cultures grow out rapidly. They also successfully cultivated Poliomyelitis virus in a medium composed of 0.5 per cent lactalbumin hydrolysate, Simms' Serum Ultrafiltrate and Earl's Balanced Salt Solution.

Bazely and Rotundo (24) in 1954, showed that human plasma fractions IV and V stimulated monkey kidney cell multiplication and could replace whole serum as a supplement to Medium 199. Yields of Poliomyelitis virus were increased.

Ginsberg and associates (25) in 1955, studied in HeLa Cell cultures the supplementary effects of various broths used for the cultivation of bacteria in a maintenance solution consisting of horse serum or ascitic fluid and balanced salt solution. Their results showed stimulation of cell multiplication with tryptose phosphate broth. These investigators believed that the stimulating supplementary effect of tryptose phospate broth was due to peptones, proteoses, or peptids.

MATERIALS AND METHODS

Seed. The organism used in this study was the Mahoney strain of Type 1 Poliomyelitis virus No. 2171 received from Eli Lilly and Company. The original source of the seed was Dr. Jonas Salk. It was stored in 1 cc. quantities in 2 cc. rubber stoppered glass ampules and held at −20°C. One seed passage was used throughout the study. The virus titer of the seed was $10^{-6.6}TCID_{50}$.

Glassware and Equipment. All glassware and equipment were carefully cleaned with detergents and rinsed several times with distilled water according to current culture practices. Sterilizing filters were chemically cleaned and rinsed with several changes of distilled water. Each filter was checked carefully with the "Bubble Test" of Rivers and Mudd (26) to assure sterilizing efficiency.

Medium Preparation. All media were made up in triple distilled pyrogen free water. All chemicals in these studies were reagent grade. All media and solutions were sterilized by filtration using Selas filters. The medium preparation is divided into four sections:

A. Preparation of Milk Hydrolysates
B. Preparation of Medium 199
C. Preparation of Maximum Test Medium
D. Preparation of Minimum Test Medium

A. *Preparation of Milk Hydrolysates.* The milk hydrolysates were selected for testing because it was noted that their amino acid composition is similar to that of Medium 199. A publication of the National Academy of Science Nutritional Research Council (27) and Sheffield Farms Inc. (28) supplied this information. The commercial products tested were selected on the basis of availability. The milk hydrolysates were made up to 10 per cent concentrations in Hank's Salt Solution.

The table which follows lists the milk products tested:

HYDROLYZED MILK PROTEINS TESTED

Commercial Product	Kind of Product	Per Cent Amino Nitrogen to Total Nitrogen
Hycase	Acid Hydrolysate of Casein (mostly amino acids and peptids)	61 %
Hycase Salt Free	Acid Hydrolysates of Casein with salts and vitamins removed (mostly amino acids and peptids)	60 %
N-Z Amine Type A	Pancreatic Digest of Casein (mostly amino acids and peptids)	53 %
N-Z Amino Type B Batch	Pancreatic Digest of Casein (mostly peptones)	39.4 %
Edamin	Pancreatic Digest of Lactalbumin (mostly amino acids and peptids)	58 %
Peptonized Milk	Trypsin Digest of Fat Free Milk (mostly peptones and polypeptids)	33.9 %
Bacto-Casitone	Pancreatic Digest of Casein (mostly amino acids and peptids)	——
Bacto-Casamino	Acid Hydrolysate of Casein (mostly amino acids and peptids)	——
N-Z Case	Tryptic Digest of Casein (mostly peptones)	40.2 %

B. *Preparation of Medium 199.* As stated previously, this medium is composed of twenty different amino acids, seventeen vitamins, two lipids, ten nucleic acid derivatives, phenol red indicator, glucose, and a number of inorganic salts in fourteen different solutions. Morgan, and Parker's (2) procedure for making this medium is followed. All solutions except Numbers 1, 2, and 9 are stored at 4°C. for a period not over thirty days. Solution No. 1, the amino acid fraction, is made fresh each time the medium is prepared. Solutions 2 and 9 form precipitates at 4°C. and thus were stored at room tmperature.

The fourteen solutions of which Medium 199 is composed are:

Solution No. 1. The Amino Acid Fraction (containing seventeen amino acids) and Balanced Salt Solution
Solution No. 2. Tyrosine and Cystine Solution
Solution No. 3. The Vitamin B Solution
Solution No. 4. The Vitamin C, Glutathione and Cysteine Solution
Solution No. 5. Biotin Solution
Solution No. 6. Folic Acid Solution
Solution No. 7. Vitamin A, D, K, Cholesterol and Tween 80 Solution
Solution No. 8. Vitamin E Solution
Solution No. 9. Adenine Sulfate Solution
Solution No. 10. Purine and Pyrimidine Solution
Solution No. 11. Ribose and Desoxyribose Solution
Solution No. 12. Muscle Adenylic Acid Solution
Solution No. 13. Adenosine Triphosphate (ATP) Solution
Solution No. 14. Ferric Nitrate Solution

C. *Maximum Test Medium.* This medium is similar to Medium 199 with the exception that the amino acid fraction is replaced with various concentrations of Edamin, a hydrolysate of lactalbumin. Concentrations of 0.1, 0.2, 0.3, 1.0, 3.0, 7.0, 10.0, 20.0 and 30.0 g/l of Edamin were tested.

D. *Minimum Test Medium.* This medium, unlike Medium 199, was made to consist of various concentrations of Edamin and Hank's Balanced Salt Solution without sodium bicarbonate. Concentrations of 1.0, 5.0 and 10.0 g/l were tested.

Solution Preparation. Reagent grade chemicals were used in all solutions, the latter being sterilized by filtration through Selas filters.

Hank's Solution. The solution was adjusted to pH 7.0 with sodium hydroxide and not buffered as in the original medium with sodium bicarbonate. Concentrations are the same as recommended by Hank and Wallace (29).

Trypsin Buffer. This solution is the phosphate buffered saline of Dulbecco and Vogt (30). It is used as a wash to remove the blood from the kidney mince that is prepared for the trypsinization process (31) for the culturing of monolayer cells.

Trypsin Solution. A solution of 0.25 per cent Difco Trypsin 1-250 in

trypsin buffer is used to trypsinize kidney cells in the preparation of cells for monolayer tissue culture.

Sodium Bicarbonate Buffer Solution. A stock solution of 2.8 per cent of sodium bicarbonate is made to buffer all media used for monolayer and suspended type cultures.

Tissue Preparation. Normal monkey kidney cells were used throughout the study. The kidneys were obtained from tuberculin-negative *Macaca rhesus* or *Macaca cynomologus* monkeys. Both monolayer and mince type tissue cultures were used. Youngner's (31) method of trypsinizing cells was used for preparing cells for the monolayer cultures.

Method for Screening the Milk Hydrolysates. A colorimetric assay test for titrating virus as outlined by Salk, Youngner and Ward (32) was adapted for use in comparing the toxicity of the milk hydrolysates to monkey kidney cells. The hydrolysate concentrations tested were from 0.1 to 50.0 g/l. The test is based on a change of the pH of the medium which is dependent on the viability of the cell and necessitates a delicately balanced buffering system, plus a color indicator. If the cells remain viable, their metabolic products will cause the medium to become acid which changes the color of the medium. Destruction or inhibition of the cells causes no color change. The test may be read visually on or after the seventh day. The indicator, phenol red, turns yellow in acid, orange in neutral and red in the alkaline state.

Method for Determining the Optimum Concentration of Test Hydrolysate. The method used to determine the optimum concentration of lactalbumin hydrolysate for cell growth was the Roller Tube Growth Test. This is a test in which trypsin dispersed cells are suspended in the test medium at 1-600 dilution, and 2.0 ml of this suspension are placed in each roller tube. The roller tube cultures are incubated at 37°C. for seven days in a stationary position after which time the cells are observed for proliferation and toxicity. After a change to fresh medium containing additional sodium bicarbonate, cultures are placed on a roller drum and incubated again for seven more days at the same temperature. On the fourteenth day the cells are again observed for viability and a majority of the tubes receive a change to fresh medium inoculated with virus. The remaining tubes also receive a medium change, the fresh medium contains no virus. All the tubes are incubated again for seven days at 37°C. At the end of this period, virus is harvested from several tubes and titrated. The uninoculated tubes are checked for toxicity and serve as controls.

The following media were tested: Medium 199, Medium 199 without the amino acid fraction, various Maximum Test Media which contained 0.1,

0.2, 0.3, 1.0, 3.0, 7.0, 10.0, 20.0 and 30.0 g/l of Edamin, and Minimum Test Media which contained 1.0, 5.0 and 10.0 g/l of Edamin.

Method Used for the Final Comparison of Virus Yields in the Test Media and Control Medium. Virus harvests from bottle cultures of monolayer tissue type cultures and suspended tissue type cultures grown in the test and control media were compared. Stoppered with specially compounded white stoppers known to be non toxic, sixteen ounce prescription type bottles were used for these cultures.

The following procedures were used for each type of culture:

A. *Monolayer cultures.* Kidney cells were prepared by the trypsin process used by Youngner (31). Test and control media buffered with 1.12 g/l of sodium bicarbonate were inoculated with cells to make a 1-600 suspension. Forty ml of each of these cell suspensions were planted in each of 10 prescription type bottles. The bottles were placed flat in a stationary position position for six to seven days at 37°C. The cell sheaths were observed at the end of this period, the old medium was drawn off and 80 ml of fresh medium, buffered with 1.68 g/l of sodium bicarbonate and inoculated with 0.02 ml of seed virus, was added to the bottles. Bottle cultures were incubated at 37°C. for three days and the virus harvested from each individual bottle and titrated.

B. *Suspended Type Cultures.* Kidney cells were prepared by mincing to approximately 1 mm size with sterile barber shears. The cells were then distributed equally into prescription type bottles with 40 ml of either test or control media containing 2.0 g/l of sodium bicarbonate. The bottle cultures were then placed on an arc type rocker and incubated at 37°C. for six days. The medium was changed and 80 ml of either test or control media containing 2.0 g/l of sodium bicarbonate were added to each of the bottles along with 0.02 ml of seed virus. The cultures were then returned to the rockers and rocked at 37°C. for four more days. The virus from each individual bottle was harvested and titrated.

Criteria for Toxicity. Normal monkey kidney cells are fusiform in shape and the cytoplasm appears hyaline. If the medium is not toxic, the tissue cultures of monolayer cells will produce luxuriant sheaths of fusiform cells with hyaline cytoplasm. When cells are exposed to toxic materials in the medium, rounded, abnormally shaped cells, with pyknotic nuclei and granular cytoplasm form. These usually die and disintegrate. When cells are deficient in nutrients the center of the cells become distended laterally, the ends of the cells shrink unevenly tending to form long thin processes and the cytoplasm remains hyaline.

Criteria for Proliferation of Cells in Monolayer Type Tissue Cultures. The trypsin dispersed cells adhere to the glass surfaces of the culture vessels

and form a luxuriant sheath of growing cells within six or seven days. The medium will become acid from the metabolic products. If the cell proliferation is inhibited by toxic materials or deficient nutrients, the cells either do not adhere to the glass surfaces or slowly form incomplete sheaths of abnormal looking cells.

Criteria for Virus Multiplication. A. *Visual.* Virus infection and multiplication is observed through the cytopathogenic phenomena reported by Enders, Weller, and Robbins (1).

B. *Numerical.* At the time of virus inoculation of the tissue cultures, samples of all virus inocula were taken and stored at 5°C. in the tissue culture medium to be tested. The viruses harvested from these cultures were then compared with the above samples to show that the viruses had multiplied and that the test medium was not inhibitory to them.

Method of Assaying Virus. Medium 199, 2 per cent horse serum, and sufficient trypsinized cells to make a 1-600 suspension of cells were dispersed in 2 ml quantities into 15x150 mm Kimble screw cap tubes. These were tightly stoppered and incubated at 36°C. in a stationary position. Cells were observed at the end of seven days and selected for virus titrations after a good monolayer of cells appeared on the glass surface of the tube. Serial ten-fold dilutions of the virus were made up. Quantities of 0.5 ml of each dilution were added to each of ten roller tubes and 1.5 ml Medium 199, containing 1.68 g/l of sodium bicarbonate, were added to all the tubes. Stoppered with specially compounded white rubber stoppers known to be low in toxicity, the tubes were placed on roller drums and incubated at 37°C. for six days. The tubes were read on the third and sixth days for cytopathogenic damage. Fifty per cent Tissue Culture Infectious Dosages ($TCID_{50}$) were calculated for each series of Media by the Reed and Munch method (33). $TCID_{50}$ is the term used by virologists to indicate the concentration of the virus inoculum required to cause cytopathogenic damage to 50% of the roller tube cultures of monolayer cells.

Method of Evaluating the Results. Fiducial limits of the mean with the probability of 95% confidence were computed for the purpose of comparing the experimental and control groups. The formulae are those from Snedecor (34).

RESULTS

The Toxicity of Various Concentrations of Hydrolysates of Milk Proteins on Monkey Kidney Cells

The following hydrolyzed products that were tested to detect toxicity are listed in the order of least toxicity to monkey kidney cells. Edamin, Hycase,

Bacto-Casamino acid, Bacto-Casitone, N-Z Amine Type A, Hycase "Salt Free," Peptonized Milk, N-Z Case, N-Z Amine Type B, and N-Z Amine Type B Batch 668. The cells were able to tolerate up to 50 g/l of Edamin, 20 g/l of Hycase and Bacto Casamino Acid, 10-30 g/l of Bacto-Casitone, and 10 g/l for the remaining products, without showing toxicity. These results are illustrated in Fig. 1 and Fig. 2 where the pH at the termination of the test is below 7.0, the hydrolysates being non-toxic to the cells at those concentrations. The cells showed toxicity in the vitamin-free product (Hycase "Salt Free") at 10.0 g/l, as compared with the cells in the similar hydrolysates containing vitamin components which were toxic at 30.0 g/l. The cells tolerated four times as much Hycase and Bacto-Casamino Acid as the

Figure 1

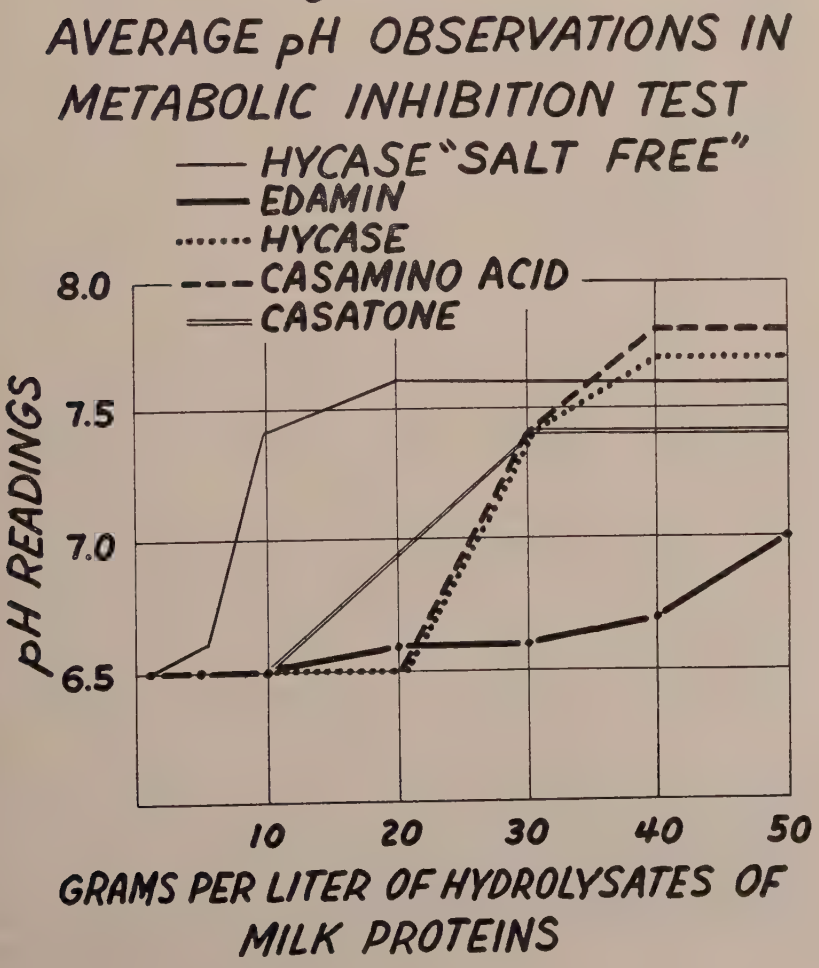
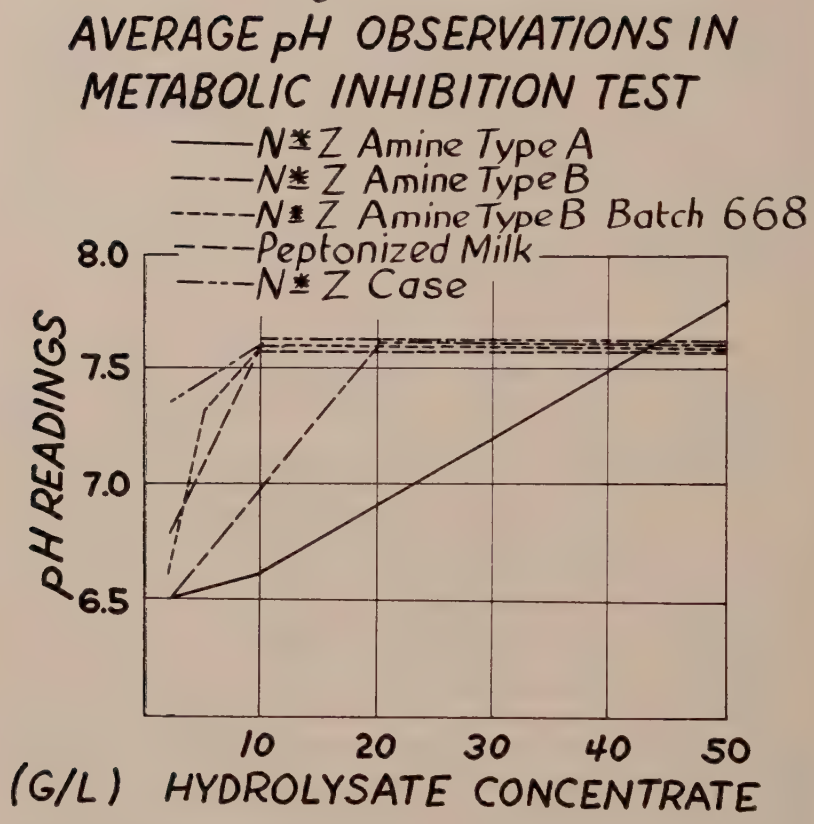


Figure 2



Hycase "Salt Free" product in 5 per cent solutions, as is shown in Fig. 1. Peptonized Milk, N-Z Case, N-Z Amine Type B, and N-Z Amine Type B Batch 668, showed toxicity to the cells in concentrations of less than 10.0 g/l, as is shown in Fig. 2. These products were found to be much more toxic to the cells than were those products such as N-Z Amine Type A and hydrolysates illustrated in Fig. 1.

Since Edamin was found to be the least toxic of all the products tested, it was chosen for further study.

The Effect of Replacing the Amino Acid Fraction in Medium 199 with Various Concentrations of Edamin on Monkey Kidney Cells and Virus Yields Obtained from These Cells

The effect of replacing the amino acid fraction in Medium 199 with Edamin results in an improved medium, since cell growth is more rapid,

cell sheaths are more luxuriant and greater virus yields are obtained than in the original medium. Cells grown in media with 1.0, 3.0, 7.0 or 10.0 g/l of Edamin the latter replacing the amino acid fraction of Medium 199, produced a good sheath of cells in less than seven days and remained in good condition for at least twenty-one days. Virus yields obtained from cultures using 1.0, 3.0, or 7.0 g/l are significantly better than Medium 199 by one log difference in virus titer.

Monkey kidney cells did not produce monolayer cultures in Medium 199 in which the amino acid fraction was left out, or in a medium containing only this fraction, or in the maximum test media containing 20 or 30 g/l of Edamin.

The results of the virus yields obtained from cultures using these media are shown in Table 1.

The Effect of Media Containing Edamin and Balanced Salt Solution on Cultures of Monkey Kidney Cells and the Virus Yields Obtained from These Cultures

The results from a limited number of tests in roller tube cultures indicated that Edamin in concentration between 1.0 and 10.0 g/l contained sufficient nutrients to stimulate monkey kidney cells to proliferate into monolayer cultures that supported good multiplication of Poliomyelitis virus. These roller tube cultures produced greater virus yields than roller tube cultures of Medium 199. (Table 2.)

Monolayer and suspended type bottle cultures using this minimal medium containing 5.0 g/l of Edamin, produced similar growths as those in Medium 199. Virus yields obtained from these cultures were no different than those obtained with Medium 199. Virus obtained from monolayer cultures have titers higher than those obtained from suspended type cultures. This difference in titer is significant with cultures using the hydrolysate medium, but it is not significant with cultures using Medium 199. (Table 3.)

Cultures using Medium 199 appeared to show some signs of degeneration after fourteen days. Cultures using the minimal medium appeared similar in appearance to Medium 199 cultures, while cultures using the maximum medium, in concentrations of Edamin from 1.0 to 7.0 g/l, showed no degenerative changes and appeared good through the twenty-first day.

DISCUSSION

The results obtained using Medium 199 as a culture medium indicate that the nutritional requirements of cells for cultivating viruses may be less exacting than those needed for maintaining cellular activity for extended periods. Cell cultures using this medium begin to show slight signs of de-

generation at fourteen days. This indicates that this medium is not complete in all the essential nutrients required by the cell for maintenance as has been shown for this medium by previous investigators. Cells proliferate to a full sheath of cells, but appear to undergo degeneration when left in Medium 199 for extended periods. Since adsorption studies on Poliomyelitis virus and monkey kidney cells have shown that the virus is adsorbed in fifteen minutes, and can produce and release virus in three to four hours, the condition of the cell at the time of virus infection would seem of more importance than the capacity of the medium to continue the support of the cell. Therefore, a knowledge of substances known to stimulate cell growth and proliferation is more useful in the development of a successful medium for virus cultivation in tissue culture than substances that will prolong the life of the cell for extended periods.

Accelerated rates of growth in cells are made possible by the introduction of additional organic materials such as amino acids, nucleo-proteins, peptids, and coenzymes. Since the anaphylactic properties of proteins and especially those from horse serum are well known, other substances known to contain growth promoting substances which lack anaphylactic properties, should be considered for media used in cultivating virusues for vaccines. Non-protein substances with these characteristics are the hydrolysates of proteins.

Contrary to the findings of the early investigators, the results here presented seem to show that the lower molecular weight products of proteins are more suitable for tissue culture of cells than are the larger molecular weight degradation products. Although this seems to be in conflict with earlier investigations, the difference in the findings may possibly be attributed to the action of enzymes known to be present in the nutrients such as serum and plasma used in the early type cultures. It would seem reasonable to assume that these enzymes brought about the breakdown of the high molecular weight products to lower degradation products which then were readily available to the cells. Thus the early work can be reinterpreted to agree with the results obtained in this investigation.

The hydrolysate of lactalbumin appeared to be tolerated by monkey kidney cells better than the hydrolysates of casein tested. Two of the more important amino acids, cystine and tryptophane, have been found to be lacking in the hydrolysates of casein. Although Rose (35) considered tryptophane but not cystine as one of the essential amino acids for animal nutrition, both of these amino acids have been found essential in tissue culture studies and bacteriological studies by many investigators. It has been shown that the vitamins, pyridine and pantothenic acid, are in lower concentrations in casein hydrolysate than in Edamin, the lactalbumin hydrolysate. Both of these vitamins have been shown to be important as growth stimulants in various plant and animal studies. These differences in the components between Edamin and the casein hydrolysates might be one explanation why Edamin

proved better than the casein hydrolysates in the tissue culture of monkey kidney cells.

The reasons for the success in obtaining greater virus yields by replacing the amino acid fraction in Medium 199 with Edamin have not been determined, but it is believed that increased virus yields are due largely to the production of a greater cell population sensitive to virus infection. Edamin, when incorporated as sole nutrient in a balanced salt solution appears to have sufficient nutrients to supply the amino acids, vitamins and other growth factors necessary for cell proliferation and is equal to the complex Medium 199 for the tissue cultivation of viruses. The minimal medium might be improved by including ribonucleic acid, desoxyribonucleic acid and by increasing the pyrimidines and purines, substances all of which have been shown in phage studies to have improved the host-virus system (36, 37, 38, 39). Ribonucleic acid is believed to be important in multiplication of the virus. The growth of the virus within a cell is accompanied by a rapid increase in ribonucleic acid content (36). Desoxyribonucleic acid is also a constituent of the new virus particles, about two-thirds of it being obtained from the components of the medium (37). According to Weed and Cohen (38) host-cell pyrimidines are utilized directly for viral synthesis and Putman (39) has shown that host purines are used intact for synthesis of virus nucleic acid. Since the Maximum medium contained ribonucleic acid, desoxyribonucleic acid, pyrimidines and purines, it would seem that these components are also important for Poliomyelitis virus in the host-virus system and it is likely that better results were obtained with the Maximum medium because of the presence of these substances.

The success in the cultivation of Poliomyelitis virus in tissue culture with the Edamin media, suggests many uses for these media. The Minimum medium might be considered for the culturing of virus for Poliomyelitis vaccine. Although further testing and approval of the government to substitute this medium for Medium 199 would be required before considering the use of Edamin Medium for vaccine purposes, the reduced cost of this medium, the simplicity of its composition, the accessibility of its components, and the virus yields obtained would probably justify the expenditures required to complete these studies. The pharmaceutical industries that manufacture Poliomyelitis vaccine could also use the Maximum Medium for testing the safety of the vaccine in tissue culture. The results suggest that use of the Edamin Maximum Medium for a culture medium would improve these tests since tissue cultures using this medium are more sensitive to virus infection and the condition of the tissue cells is so much better than that of cells grown in Medium 199. These media might also be used in pH Test, which is used for the potency testing of the vaccine, since it would eliminate the use of horse serum which is sometimes toxic to the cells and causes nonspecific reactions which hamper the reading of the potency test results.

TABLE 1

VIRUS YIELDS FROM ROLLER TUBE CULTURES USING MAXIMUM TEST MEDIA

HYDROLYZATE CONTENT IN MEDIA	NUMBER OF REPLICAS	FIDUCIAL LIMITS ($X \pm S \times t\ 0.05$)
0.1	4	5.5 ± 0.91
0.2	4	6.2 ± 0.59
0.3	4	6.6 ± 0.67
1.0	9	7.3 ± 0.19
3.0	2	7.6 ± 1.27
7.0	5	7.3 ± 0.35
10.0	6	6.7 ± 0.59
20.0	3	NO CELLS
30.0	3	NO CELLS
CONTROL—199 MEDIUM	10	6.5 ± 0.27

TABLE 2

VIRUS YIELDS FROM ROLLER TUBE CULTURES USING MINIMUM TEST MEDIA

HYDROLYZATE CONTENT IN MEDIA (GRAMS/LITER)	NUMBER OF REPLICAS	FIDUCIAL LIMITS $X \pm S \times t\ 0.05$
1.0	2	6.6 ± 0.64
5.0	2	7.7 ± 1.27
10.0	2	7.5 ± 1.93
CONTROL MEDIA		
199 Medium	10	6.5 ± 0.27
199 Medium Without Amino Acid Fraction	3	No Cells

TABLE 3

VIRUS YIELDS FROM BOTTLE CULTURES

NUTRIMENT	TYPE OF CULTURE	NUMBER OF REPLICAS	FIDUCIAL LIMITS ($X \pm S \times t\ 0.05$)
1. Minimal Test Medium	M	10	7.7 ± 0.23
2. Medium 199	M	5	7.4 ± 0.36
3. Amino Acid Fraction in Medium 199	M	12	No Cells
4. Minimum Test Medium	S	14	7.1 ± 0.28
5. Medium 199	S	10	7.1 ± 0.24

M—Monolayer Cultures

S—Suspended (Mince) Type Cultures

SUMMARY

1. A study was made of ten different hydrolyzed products of milk proteins for their toxicity to monkey kidney cells. Edamin, a hydrolysate of lactalbumin, proved to be the least toxic. The cells could tolerate two and a half times as much Edamin as Hycase and Casamino acids, both hydrolysates of casein which were the next two showing least toxicity.

2. It was shown that Edamin could provide sufficient nutrients to replace the amino acid fraction in Medium 199. Cells in media containing 1.0, 3.0, 7.0 and 10.0 grams of Edamin per liter, produced a good sheath of cells in monolayer tissue type cultures in seven days and remained in good condition, with little or no toxicity, for twenty-one days.

3. Poliomyelitis virus yields obtained from monkey kidney cells grown in media similar to Medium 199, but in which the amino acid fraction of Medium 199 has been replaced with 1.0, 3.0 or 7.0 g/l Edamin, were significantly better than Medium 199 in monolayer tissue type cultures.

4. Roller tubes of monolayer cultures grown in media containing 5.0 or 10.0 g/l Edamin in Hank's Salt Solution, produced Poliomyelitis virus yields that were significantly better than Medium 199.

5. Bottle cultures, using a minimal test medium containing only 5.0 g/l Edamin in Hank's Salt Solution, produced suspended type and monolayer tissue cultures that produced Poliomyelitis virus yields equal to those obtained with Medium 199.

6. The practical aspects and the implications of these findings to cell nutrition are discussed.

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ERYTHROMYCIN, STUDIES OF ITS MODE OF ACTION

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Relatively little is known of the mode of action of erythromycin. This antibiotic is widely used in the treatment of infectious diseases and has undoubtedy prolonged the lives of thousands of people. It would seem axiomatic that more knowledge of the mode of action would serve as a foundation for more effective clinical use.

Although Pittenger et al. (1) and Haight and Finland (2) had tested some organic and inorganic compounds for reversal of the inhibitory action of erythromycin, it seemed profitable to use a more informative method. Such tests have usually been carried out in liquid media using a single concentration of the compound in question and one level of the inhibitory substance. It was felt that by the use of agar plates, seeded with a sensitive organism, a solution of the test compound could be introduced on a filter paper disk. Through diffusion an infinite variety of concentrations of the test substance would be present in concentric zones around the filter paper disk. The antibiotic could be added either in the bacteriostatic threshold concentration to the liquid seeded agar medium before pouring the plate, or added on filter paper disks close to the test compound disks on the hardened agar surface. After incubation the zones of growth or inhibition could be interpreted in terms of reversal or potentiation of the inhibition of erythromycin and toxicity of the test compound.

Brown and Emerson (3) found that pantothenic acid, beta-alanine and carnosine reversed the inhibitory action of erythromycin towards the *gravis* strain of *Corynebacterium diphtheriae*. No confirmation has been made to date by other investigators. It would seem desirable not only to attempt to confirm their work but also to look for a similar situation in other organisms. It appeared that organisms in which such an effect would be most apparent would be those which require either pantothenic acid or beta-alanine for growth. In this category *Lactobacillus casei* and *L. arabinosus* require pantothenic acid for growth and numerous strains of yeast require either pantothenic acid or beta-alanine. Since erythromycin is used in the treatment of diseases caused by *Diplococcus, Micrococcus* and *Streptococcus,* it seemed pertinent to use those organisms to test for the reversal by pantothenic acid or beta-alanine of the inhibition due to erythromycin. Finally, it seemed desirable to determine whether the resistance of highly resistant strains of *Mi-*

crococcus pyogenes var. *aureus* is due to high production of pantothenic acid.

Many organisms which are resistant to penicillin are resistant by virtue of their production of penicillinase, an enzyme capable of destroying penicillin. This enzyme has been found to be extracellular and intracellular in normally resistant organisms and in organisms in which the resistance has developed (4). As one of the possible mechanisms of resistance to erythromycin, it seemed obvious that an attempt should be made to find erythromycinase. Haight and Finland (2), using many strains of the highly resistant gram-negative organisms, could find no extracellular erythromycinase. Since resistance in staphylococcal infections has become a serious problem, it appeared of value to determine the presence or absence of intracellular or extracellular erythromycinase in the two highly resistant strains of *Micrococcus pyogenes* var. *aureus* available.

One possible mode of action of erythromycin would be its interference with the functioning of one or more enzymes necessary to the growth or reproduction or action of the sensitive organism. Stone et al. (5) recently reported that staphylococci which were highly resistant to erythromycin in vitro were still sensitive when treated in vivo in mice. Since the pathogenicity of the staphylococcus is closely associated with coagulase production, one explanation for this phenomenon would be an inhibition of coagulase by the antibiotic.

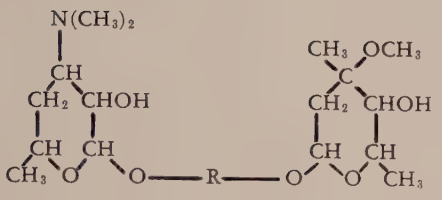
REVIEW OF THE LITERATURE

Erythromycin has the formula $C_{37}H_{67}NO_{13}$ with a structure approximating that shown in Fig. 1 and a molecular weight of 734. It is a base with a pK 8.6. It is slightly soluble in water and more readily soluble in organic solvents. A pH of less than 4 or higher than 10 is highly destructive (6, 7). Degradation studies have led to the identification of large fragments, including erythralosamine, $C_{29}H_{49}NO_8$, and cladenose, $C_8H_{16}O_4$ (Fig.1) (7). One fraction of erythralosamine is desosamine, $C_8H_{17}NO_3$ (Fig. 1) (7). A large fragment, dihydroerythronolide, $C_{21}H_{41}O_8$, has been described (Fig. 1) (8).

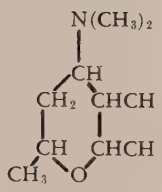
Erythromycin appears to be a member of a group of antibiotics which includes: albomycetin ($C_{32}H_{54}NO_9$, mol. wt., 596) (9), amaromycin (10), angolamycin ($C_{49-51}H_{87-91}NO_{18}$, mol. wt., 977-1005) (11), carbomycin ($C_{41}H_{67}NO_{16}$, mol. wt., 830) (12, 13), carbomycin B ($C_{41-42}H_{67-69}NO_{15}$, mol. wt., 814-828) (14), celesticetin ($C_{24}H_{36-40}N_2O_9$, mol. wt., 496-500) (15), erythromycin B ($C_{37}H_{71}NO_{12}$, mol. wt., 721) (16), griseomycin (17), leucomycin ($C_{27}H_{42}NO_{10}$, mol. wt., 540) (18), methamycin ($C_{25}H_{43}NO_7$, mol. wt., 470) (19), narbomycin ($C_{28}H_{47}NO_7$, mol. wt., 510) (20), antibiotic PA-105 (21), pikromycin ($C_{25}H_{43}NO_7$, mol. wt., 477), (22), rhodomycin (23) and spiromycin (24).

In general the members of the erythromycin antibiotic group exhibit

FIGURE 1



a



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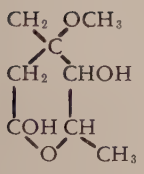

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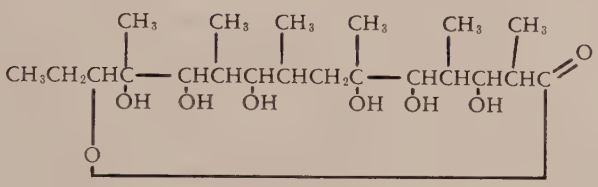

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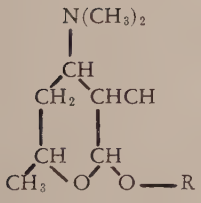

e

a. erythromycin, b. desosamine, c. cladenose, d. dihydroerythronolide, e. erythralosamine (R is dihydroerythronolide minus 2H; R' is dihydroerythronolide minus $2H_2O$).

similar antibacterial and antiviral spectra. The resemblance of the spectra of carbomycin (12), celesticetin (25), erythromycin B (26), griseomycin (17), antibiotic PA-105 (21) and spiromycin (24) to that of erythromycin has been noted by other investigators. Organisms showing moderate to high sensitivity to erythromycin include the following genera: *Bacillus, Brucella, Clostridium, Corynebacterium, Diplococcus, Hemophilus, Micrococcus, Mycobacterium, Neisseria, Rickettsia, Streptococcus, Vibrio,* lymphogranuloma virus and meningopneumonitis virus. In general the gram-negative organ-

isms, *Escherichia, Proteus, Pseudomonas, Salmonella* and *Shigella* show high resistance. There are variations in the degree of effectiveness of the different members of this antibiotic family. Carbomycin, for example, is usually less effective than erythromycin. There are some marked differences. Amaromycin, for example, inhibits *Brucella abortus* with 1.56 mcg/ml (10) while erythromycin does not inhibit the organism with 1000 mcg/ml (17) even though it is quite active against other members of the genus (6).

Cross-resistance has been demonstrated between erythromycin and erythromycin B (27), carbomycin (28), celesticetin (25), griseomycin (17), antibiotic PA-105 (21), and spiromycin (24). Cross-resistance evidence suggests that leucomycin is closely related to carbomycin and somewhat less closely related to erythromycin (29).

The chemical structure desosamine has been demonstrated in erythromycin (7), erythromycin B (16), pikromycin (23) and narbomycin (20). Carbomycin and carbomycin B contain the very similar desoxydimethyl amino sugar, mycaminose (14). In rhodomycin the order in which the radicals are attached to the desosamine ring has been changed (23). One of the radicals attached to the ring structure in desosamine is a dimethyl amine. It is interesting to note that albomycin (9), angolamycin (11), carbomycin (12), carbomycin B (14), erythromycin (7), erythromycin B (16), leucomycin (18), methamycin (19), narbomycin (20) and pikromycin (22) all have but a single nitrogen atom.

The literature on other members of the erythromycin antibiotic family, reviewed here, indicates no mode of action.

The effect of various inorganic and organic substances on the bacteriostatic action of erythromycin has been investigated. Pittenger et al. (1), working with *Micrococcus pyogenes* var. *aureus* in vitro, found that various concentrations of Cl^-, NO_3^-, SO_4^-, Na^+, K^+, Mg^{++}, Ca^{++}, Mn^{++}, Cu^{++}, Co^{++}, Fe^{++}, Zn^{++}, Na-thioglycollate, cysteine-HCl, acetate, fumarate, alpha-glutarate, malate, oxalactate, pyruvate, succinate, citrate and vitamin mixtures (riboflavin, thiamin, biotin, niacin, p-aminobenzoic acid, calcium panthothenate, pyridoxine, pyridoxal, pyridoxamine and folic acid) had neither appreciable potentiation or reversal of the bacteriostatic ability of erythromycin. Haight and Finland (2) found that various concentrations of NaCl, dextrose, Na-thioglycollate, cysteine-HCl, semicarbozide, urea, glutamic acid, p-aminobenzoic acid and pteroylglutamic acid had no significant effect on the in vitro inhibition of *Bacillus cereus* no. 5 by erythromycin. They also reported that various concentrations of the sodium salts of citric, pyruvic, acetic, lactic, fumaric and succinic acids and the enzyme penicillinase had no effect on the inhibition of *Streptococcus* no. 98. Human sera at various levels had no effect on the antibiotic's inhibition of *Sarcina lutea.* Brown and Emerson (3) reported that 500 mcg/ml beta-alanine or calcium pantothenate, equivalent to 200 mcg/ml beta-alanine, permitted growth of

Corynebacterium diphtheriae in 70 times the original minimal inhibitory concentration of erythromycin. L-carnosine, equivalent to 100-400 mcg/ml of beta-alanine, reversed the inhibition of up to 30 times the minimal inhibitory concentration of the antibiotic.

Haight and Finland (2) found a 530 and 800-fold increase of the in vitro inhibitory activity of erythromycin against *Streptococcus* no. 98 and *Sarcina lutea,* respectively, when the pH was increased from 5.6 to 8.5. Pittenger et al. (1) found a 30-fold increase in the bacteriostatic action against *Micrococcus pyogenes* var. *aureus* with the increase of pH from 6.5 to 8.5. They also noted that an apparent potentiating effect of phosphates on the activity of erythromycin was actually due to the effect of the buffering action on pH. Pittenger et al. (1) suggested that the free base had much more activity than the salt of the antibiotic and that the increase of activity as the pH increased was due to release of the free base.

Haight and Finland (2) found that a change in the pH by replacing the oxygen with carbon dioxide did not affect the bacteriostatic action of erythromycin against *Streptococcus* no. 98, *Streptococcus* C203, *Sarcina lutea, Streptococcus viridans, Diplococcus pneumoniae* type III, *Staphylococcus* no. 195 or an enterococcus.

Resistance has been developed in sensitive strains in the laboratory. Pittenger et al. (1) found that *Micrococcus pyogenes* var. *aureus* increased its resistance to erythromycin about 300-fold in 25 serial transfers in the presence of the antibiotic. The development of resistance to erythromycin was slightly less rapid than the development of resistance to penicillin and much less rapid than the development of resistance to streptomycin under the same conditions. No organism which had become nutritionally dependent on erythromycin was found. Hsie and Kotz (30), however, developed a strain of *M. pyogenes* var. *aureus* which had become nutritionally dependent on carbomycin.

Haight and Finland (2) attempted to determine whether species which are naturally resistant to erythromycin produce erythromycinase. Using the method of Gots (31) numerous strains of *Pseudomonas aeruginosa, Aerobacter aerogenes, Klebsiella pneumoniae, Escherichia coli, Proteus vulgaris, Salmonella* and *Shigella* were checked for the production of an extracellular erythromycinase which would destroy erythromycin which was present in an amount just inhibitory to the test organism, *Sarcina lutea.* No evidence of destruction of the antibiotic was found. By a second method, suspensions of *Proteus vulgaris* and *Escherichia coli* organisms had no destructive effect on a solution of erythromycin over a 72-hour period.

Obviously, there are present in nature mechanisms for the destruction of this antibiotic. The erythromycin content of plasma in vivo drops rapidly without a corresponding amount being excreted in the urine or otherwise being accounted for (2). One scrap of evidence of its metabolism has been

found. Des-N-methyl erythromycin has been isolated from the bile of dogs (32).

Stone et al. (5) found that coagulase-positive strains of *Micrococcus pyogenes* var. *aureus* which had been isolated from human cases showed large variation in in-vitro sensitivity but showed small variation in in-vivo sensitivity to erythromycin. Inhibition in vitro was caused by 0.195 mcg/ml erythromycin for strain no. 3055A, 10,000 mcg/ml, for strain no. 3066A, and 5,000 mcg/ml, for strain no. 3072A. In 20 to 25 g Swiss mice, the effective dose of erythromycin 50 per cent of the time was 18 mcg for strain no. 3055A, 22 mcg for strain no. 3066A and 116 mcg for strain no. 3072A. Of significance, in interpretation of this work, is the widely accepted belief that coagulase production is the best indication of pathogenicity in strains of *M. pyogenes* var. *aureus* (33). Boniece et al. (34) found that erythromycin was effective in reducing the coagulase activity of numerous strains of *M. pyogenes* var. *aureus*.

In one of the few papers on the effect of erythromycin on enzymes, Jacobson and Ayevedo (35) reported that erythromycin did not inhibit fumerase, lipase, cholinesterase and thiaminase from animal sources or the fermentation of alcohol by yeast.

MATERIALS AND METHODS

Materials

ORGANISMS:

Bacillus subtilis, spore suspension, strain X12.1, R. C. Pittenger
Corynebacterium diphtheriae, strain X166, R. C. Pittenger, strain Toronto, McClain
Diplococcus pneumoniae, strain type I, Park
Lactobacillus casei, no. 7469, American Type Culture Collection
Micrococcus pyogenes var. *aureus,* strain no. 3055, strain no. 3066, strain no. 3067, strain no. 3074, all from Boniece
Proteus vulgaris, strain no. 9484, American Type Culture Collection
Pseudomonas aeruginosa, strain X239, R. C. Pittenger
Streptococcus pyogenes, C203, strain no. 8868, American Type Culture Collection
Saccharomyces cerevisiae, "Gebrüder Mayer," strain no. 7752, "Old Process," strain no. 7753, "Fleischmann's Baker's," strain no. 7754, American Type Culture Collection

MEDIA:

Brain heart infusion (Difco) used with *Corynebacterium*
Hydrolyzed casein medium (36) used with *Micrococcus*
Mycin assay ager (Difco) used with *Bacillus subtilis*
Tryptose (Difco), 1 per cent, in nutrient broth (Difco) used with *Corynebacterium*
Pantothenic acid assay medium (Difco) used with *Lactobacillus*
Seed agar (Baltimore Biological Laboratories) used with *Micrococcus, Proteus* and *Pseudomonas*
Tryptose medium (37) used with *Diplococcus* and *Streptococcus*
Yeast synthetic medium (38, 39) used with yeast

INSTRUMENTS:

Laboratory Model G, Beckman pH Meter

Junior Spectrophotometer, Model 6A, Coleman Instruments, Inc.

OTHER MATERIALS:

Alumina A-303, Aluminum Company of America

Discs of very pure, highly absorbent paper for the assay of penicillin and other antibacterial substances, $\frac{1}{2}$-in (12.7 mm) diam, No. 740-E, Carl Schleicher and Schuell

Erythromycin standard solution, 100 mcg/ml. One hundred mg 'Ilotycin' (erythromycin, Lilly), lot no. 652282, was dissolved in 100 ml 1 per cent sodium and potassium phosphate solution adjusted to a pH of 7.0 (Hartman-Leddon Co.) and filtered through a Seitz filter. Solution was made up fresh at least once a month and stored in a refrigerator until used.

"Tes-Tape," a paper impregnated with glucose oxidase and horse-radish peroxidase, Eli Lilly Company

Screening of Compounds to Determine Reversal or Potentiation of Action of Erythromycin

Method of Screening. Work was begun using seeded agar plates to which erythromycin was added on a centrally located paper disk. Solutions of compounds to be tested were added to paper disks which had been placed on the agar. Part of the disk would cover agar in which the antibiotic would diffuse from the erythromycin disk and would be expected to inhibit growth. Part of the disk would cover agar where no inhibition would be expected to occur.

It was decided that seeded plates, in which a bacteriostatic threshold level of erythromycin was present, would constitute a sensitive means of demonstrating any reversal action that a compound might have. In this event the disk to which an active compound had been added would be surrounded by a circular zone or a halo in which growth of the test organism took place. The diameter of the zone would be related to diffusability of the compound as well as to its inherent activity.

Micrococcus pyogenes var. *aureus* no. 3055, a strain sensitive to several antibiotics, and spores of *Bacillus subtilis* were used for these tests. Synthetic media were tried first and found unsatisfactory. Seed agar (BBL) was used with *M. pyogenes* and mycin agar (Difco) was used with *B. subtilis*. Ten ml of heated liquid agar was poured into a Petri dish. After the agar base had hardened, 5 ml additional liquid agar containing 0.5 ml of a 1/100 dilution of either organism and, when indicated, containing 0.5 ml of the appropriate concentration of erythromycin, were layered in on top. When *M. pyogenes* was used, 50 mcg of the antibiotic was either incorporated in the seeded agar or added to the paper disk. When *B. subtilis* was used, 10 mcg of the antibiotic was used. The antibiotic was added to the paper disk in 0.05 ml quantities; solutions of test compounds were added in 0.07 ml quantities. To prevent absorption of moisture from the medium by the paper disks, solutions were added to the disks immediately after they had been placed on

the agar. Plates were incubated at 37°C. for 24 hours and then examined for growth.

Compounds Screened. A large number of compounds were screened for effect on the inhibitory action of erythromycin against *Micrococcus pyogenes* var. *aureus* and the spores of *Bacillus subtilis*, using both techniques. Compounds and their per cent concentration in solution were as follows:

amino acids: DL-alpha-alanine, 1 and 10; beta-alanine, 0.1 and 10; L-arginine, 1; DL-aspartic acid, 1 and 2; hydrolyzed casein (vitamin-free), 10; DL-citrulline, 1; creatine, 1; L-cysteine-HCl, 1 and 10; ethionine, 1; L-glutamic acid, 1 and 2; L-histidine-HCl, 1 and 5; DL-isoleucine, 2; L-leucine-HCl, 1; DL-lysine-HCl, 1; DL-methionine, 1; norleucine, 1; DL-phenylalanine, 1; L-proline, 1; DL-serine, 1; DL-threonine, 1; DL-tryptophan, 1; DL-valine, 2

purines: adenine sulfate, 1; guanine-HCl, 0.7; uracil, 1; xanthine, 1 (All purines were brought into solution with NaOH.)

organic acids: citric acid, 1 and 10; oxalic acid, 1 and 10

vitamins: acetyl choline-HCl, 10; ascorbic acid, 1; beta-alanine, 0.1 and 10; biotin, 0.000,04; Ca-pantothenate, 0.1 and 1; inositol, 0.1; niacin, 0.06; p-aminobenzoic acid, 1; pimelic acid, 0.025; pyridoxine, 0.12; thiamine-HCl, 0.1; yeast extract, 10

carbohydrates: dextrose, 10; lactose, 10; levulose, 10; D-maltose, 1; mannose, 1; mannitol, 10; raffinose, 10; rhamnose, 2; ribose, 1; sucrose, 1 and 10; xylose, 10

miscellaneous organic compounds: adenylic acid, 1; L-asparagine, 1 and 10; beef extract, 10; betaine, 1; creatinine, 1; desosamine, 10; D-glucosamine, 1; glutathionine, 1; glycerol, 10; mercaptoethyl amine, 10; mucin, 5; Na-thioglycollate, 1; tetra sodium salt of ethylene diamine tetra acetic acid, 9.4

inorganic compounds: $FeSO_4.7H_2O$, 0.1 and 10; KCl, 0.1; $ZnSO_4.7H_2O$, 0.1; $TlSO_4$, 0.1; H_3BO_3, 0.1; NaH_2PO_4 and Na_2HPO_4 (pH 7.0), 10

Hydrogen Ion concentration. The pH was determined for all samples screened which had not been previously discarded.

Since some of the compounds which reversed erythromycin were found to have pH's of 4.0 or less, the pH's of the solutions of seven of these compounds, adenylic acid, ascorbic acid, L-aspartic acid, L-cysteine-HCl, L-glutamic acid, glutathione and L-histidine-HCl, were brought up to the range of 6.0 to 7.0. Solutions of these neutralized compounds were added to paper disks on seeded plates containing a bacteriostatic amount of the antibiotic and checked for reversal of the action of the antibiotic.

L-Cysteine-Hydrochloride. This compound was tested for reaction with erythromycin under mildly acid conditions. A 0.2 per cent L-cysteine-HCl solution was made in erythromycin standard solution and the pH adjusted to 5.2. As a control, erythromycin standard solution was adjusted to a pH of 5.2. The cysteine-erythromycin and control solutions were heated in a 56°C. water bath for 1 hour. Fifteen ml of each solution were filtered through a Seitz filter and compared, using paper disk assay techniques with both test organisms. The diameters of the zones of inhibition were measured and compared as an indication of comparative potency. The remainder of the cysteine-erythromycin and control solutions were stored in a refrigerator at 6°C. for

about 72 hours. The solutions were then filtered and comparative potencies again determined.

Organic and Inorganic acids. In view of the data which showed that compounds which reverse the action of erythromycin were acidic, additional acid substances were tested in the same manner as the original screening. Substances tested were alanine 2 per cent and HCl 1 per cent, glycine 2 per cent and HCl 1 per cent, HCl 1 per cent, lactic acid 1 per cent, malic acid 1 per cent, H_2SO_4 1 per cent and tartaric acid 1 per cent.

Revival. Finally, chemicals with the ability to reverse the action of erythromycin, including acidic substances, were checked for revival of organisms which had been inhibited by erythromycin. Agar plates seeded with either test organism and containing bacteriostatic concentrations of erythromycin were incubated at 37°C. for 24 hours. Solutions of test compounds were added to paper disks on the surface of the agar. The plates were incubated an additional 48 hours and then examined for revival of organisms.

Investigation of the Reversal by Calcium Pantothenate and Beta-Alanine of the Inhibitory Effects of Erythromycin on Several Species of Bacteria

Lactobacillus casei. L. casei was used to determine if Ca-pantothenate and beta-alanine have the ability to reverse the inhibition caused by erythromycin. The procedure followed was that of the Difco Manual (40). Amounts of Ca-pantothenate added to the tubes were equivalent to 0, 0.02, 0.2, 2 and 20 mcg/ml of the final volume. Four tubes containing each level of Ca-pantothenate received 0, 0.04, 0.2 and 1 mcg erythromycin per ml of the final volume, respectively (Table 1). Distilled water was added to bring the volume to 5 ml. Five ml pantothenic acid assay medium was added. The culture tubes were sterilized by autoclaving and then inoculated with a suspension of washed *L. casei.* The cultures were incubated at 37°C. for 72 hours. The pH was determined for each culture. An interpretation was made on the basis of pH as an indication of growth and acid production. This test was repeated. Subsequently, a test was set up in which all tubes received 0.2 mcg/ml Ca-pantothenate. Amounts of beta-alanine added to tubes were 0, 0.2, 2, 20 and 200 mgc/ml of the final volume. Four tubes containing each level of beta-alanine received 0, 0.2, 2, and 20 mcg erythromycin per ml of the final volume, respectively (Table 2).

Saccharomyces cerevisiae. Three strains of *S. cerevisiae,* "Old Process," "Fleischmann's Baker's" and "Gebrüder Mayer," were checked for their requirements of Ca-pantothenate. Using the synthetic medium of Williams et al. (38, 39) "Gebrüder Mayer" gave the greatest response to the addition of Ca-pantothenate to the medium. A test was set up with all tubes in duplicate. Amounts of Ca-pantothenate added to the tubes were equivalent to 0, 0.005, 0.01, 0.02, 0.03, 0.04 and 0.05 mcg/ml of the final volume. Eight

tubes containing each level of Ca-pantothenate received 0, 1, 10 and 100 mcg erythromycin per ml of the final volume for each pair, respectively (Table 3). After inoculation, tests were incubated at room temperature for 5 days. Readings of optical density were made at a wave length of 550 lambdas with a spectrophotometer. Subsequently, another test was run in which amounts of beta-alanine added to the tubes were equivalent to 0, 0.2, 0.4, 0.6, 0.8 and 1 mcg/ml of the final volume. Eight tubes containing each level of beta-alanine received 0, 1, 10 and 100 mcg erythromycin per ml of the final volume for each pair, respectively (Table 4).

Micrococcus pyogenes var. *aureus*. *M. pyogenes* var. *aureus*, strains no. 3055 and no. 3067, were investigated using hydrolyzed casein medium (36). Amounts of Ca-pantothenate added to the tubes were equivalent to 0, 0.1, 1 and 10 mcg/ml of the final volume; amounts of beta-alanine, were equivalent to 0, 10, 100 and 1000 mcg/ml. For each strain a set of four tubes at each level of Ca-pantothenate or beta-alanine received 0, 0.1, 0.2, 0.5, 1 and 2 mcg erythromycin per ml of the final volume, respectively (Table 5). After inoculation, cultures were incubated at 37°C. for 5 days. Growth was determined by a visual estimate of the turbidity. Very heavy growth was given a value of 4; heavy growth, 3; moderate growth, 2; slight growth, 1; and no growth, 0.

Streptococcus pyogenes and Diplococcus pneumoniae. *S. pyogenes* C-203 and *D. pneumoniae* Park type 1 were investigated using tryptose medium (37). Amounts of Ca-pantothenate added to the tubes were equivalent to 0, 0.1, 1 and 10 mcg/ml of the final volume; amounts of beta-alanine, were equivalent to 0, 10, 100 and 1000 mcg/ml. For each organism a set of four tubes containing each level of Ca-pantothenate or beta-alanine received 0, 0.005, 0.01, 0.02 and 0.04 mcg erythromycin per ml of the final volume, respectively (Table 6). After inoculation, cultures were incubated at 37°C. for 4 days. Growth was determined by a visual estimate of the turbidity.

Corynebacterium diphtheriae. *C. diphtheriae* strains X-166 and Toronto were investigated using a medium of 1 per cent Tryptose (Difco) added to nutrient broth. Amounts of Ca-pantothenate added to the tubes were equivalent to 0, 0.1, 1 and 10 mcg/ml of the final volume; amounts of beta-alanine, were equivalent to 0, 10, 100 and 1000 mcg/ml. For each strain four tubes containing each level of Ca-pantothenate or beta-alanine received 0, 0.004, 0.0008, 0.016 and 0.032 mcg erythromycin per ml of the final volume, respectively (Table 7). It was necessary to use a large inoculum to get satisfactory growth. One drop of undiluted 5-day culture was used. After inoculation, the cultures were incubated at 37°C. for 9 days. After vigorous shaking, the growth was determined by a visual estimate of turbidity.

Pantothenic Acid Production by Resistant Strains of Micrococcus pyogenes var. aureus. Two resistant strains of *M. pyogenes* var. *aureus*, no. 3066 and no. 3074, and sensitive strain no. 3055 were grown in hydrolyzed casein

medium without the presence of pantothentic acid and beta-alanine. After inoculation, the cultures were incubated at 37°C. for 24 hours. A 10 ml aliquot of each culture was autoclaved at 15 lb. steam pressure for 15 minutes. To each culture was added 10 ml 4 per cent acetic acid, 2 ml N/1 NaOH and 1 g mylase. The tubes were placed in a 50°C. water bath for 24 hours. The pH was brought to 7.0. These samples were assayed for pantothenic acid using *Lactobacillus casei* in the manner earlier described.

Investigation of the Presence of Intracellular or Extracellular Erythromycinase

Gots' Method, Extracellular Erythromycinase. Using the method of Gots (4), *Proteus vulgaris, Pseudomonas aeruginosa* and resistant strains of *Micrococcus pyogenes* var. *aureus* were checked for the production of extracellular erythromycinase. Seed agar (BBL) plates were prepared which were seeded with sensitive strains *M. pyogenes* var. *aureus*, no. 3055 and no. 3067, and which contained 1.4 mcg erythromycin per ml. The plates were streaked with the above mentioned resistant organisms. The plates were incubated at 37°C. for 24 hours and examined for the growth of the seeded organisms in the agar.

Breakdown of Erythromycin in Solution by Extracellular Erythromycinase. Resistant strains of *Micrococcus pyogenes* var. *aureus*, no. 3066 and no. 3074, were used in a second check for the production of an extracellular erythromycinase. It had been previously determined that these strains could grow in the presence of 200 mcg erythromycin per ml. Cellophane tubes containing 5 ml hydrolyzed casein medium (36) were immersed in 25 ml of the same medium in 40 mm diam culture tubes. The dialyzing tubes were inoculated with resistant strains no. 3066 and no. 3074, respectively, and 100 mcg erythromycin per ml was added to the medium surrounding the dialyzing tubes. A third tube was inoculated with sensitive strain no. 3055, no antibiotic being added to the medium. A fourth tube was prepared in which erythromycin was added but no inoculation was made. After 72 hours incubation at 37°C., dilutions were made of the media outside of the dialyzing tubes. The dilutions were made in hydrolyzed casein medium in 2-fold steps from 1:2 to 1:64. The diluted medium was then inoculated with sensitive strain no. 3055 and incubated at 37°C. for 2 days. The presence or absence of growth in the dilutions was noted.

Solvent Treatment of Cells to Release Intracellular Erythromycin. Abraham and Chain (41) originally found penicillinase as an intracellular enzyme by grinding up cells of *Escherichia coli*. Kirby et al. (42) found that the treatment of *Micrococcus* cells by fat-removing solvents, acetone and ether, was a satisfactory means of making intracellular penicillinase available. Gilson and Parker (43) found that by using the solvents at temperatures of −20°C.

they had less inactivation of the enzyme. Adapting the method of Gilson and Parker to the facilities available, resistant strains, no. 3066 and no. 3074, of *Micrococcus pyogenes* var. *aureus* were checked for the production of erythromycinase.

The entire surface of plates of seed agar (BBL) was inoculated with a suspension of the resistant *M. pyogenes* strains. Two plates were inoculated with each strain. After 24 hour incubation at 37°C., 3 ml sterile saline solution were added to each plate. The organisms were suspended in saline solution. About 5 ml of suspension of each strain were harvested and added to a centrifuge bottle. Each centrifuge bottle was placed in a beaker of alcohol which had been cooled at −20°C. Twenty-eight ml of acetone, cooled to −20°C., were gradually added with shaking. The suspension in the centrifuge bottle was maintained within the range of −10°C. to −20°C. After 75 minutes with frequent shaking, the acetone was removed with suction. An additional 25 ml of −20°C. acteone was added to the residue and the suspension maintained at −10°C. to −20°C. with frequent shaking for 75 minutes. The acetone was again removed with vacuum. This time, 25 ml of −20°C. ether were added to the residue and the suspension maintained at −10°C. to −20°C. with frequent shaking for 75 minutes. The ether was removed with suction. The residue in the centrifuge bottle was dried by evacuation to a pressure of below 100 microns Hg for 1 hour.

Erythromycin was added to hydrolyzed casein medium at dilutions from 1 mcg to 200 mcg per ml of final volume. One series of tubes served as controls, a second series received a suspension of no. 3066 treated cells; a third series received a suspension of no. 3074 treated cells. All tubes were inoculated with a suspension of the sensitive no. 3055 strain, excepting sterility controls. Since sterility controls on inoculation indicated that viable *Micrococcus cells* were present in the residues, another plan was necessary to demonstrate the presence or absence of erythromycin.

An erythromycin standard solution was brought in contact with the defatted cells, sterilized by filtration, and checked for destruction of the antibiotic. A tube containing 3 ml of erythromycin standard solution, 1000 mcg of the antibiotic per ml, received 1.7 mg of defatted no. 3066 cells; a second tube received 4.1 mg of defatted no. 3074 cells; a third tube received no cells and acted as a control. All tubes were left at room temperature for 48 hours. Twelve ml distilled water were added to each tube and the contents then filtered through a Seitz filter. Dilutions of the filterates added to hydrolyzed casein medium were equivalent to 0, 0.10, 0.39, 1.56, 6.25, 25 and 100 mcg of erythromycin originally present per ml of final volume. All tubes were inoculated with the sensitive strain no. 3055 of *M. pyogenes* var. *aureus*. After incubation at 37°C. for 4 days, the tubes were examined for growth.

Physical Destruction of Cell Wall to Release Intracellular Erythromycinase. According to Hugo (44) and McIlwain (45), a very satisfactory

method of preparing cell-free enzymes is by the physical rupturing of the cell wall with fine abrasives. Resistant strains no. 3066 and no. 3074 of *Micrococcus pyogenes* var. *aureus* and the resistant organisms *Proteus vulgaris* and *Pseudomonas aeruginosa* were treated in this manner to determine the presence of intracellular erythromycinase.

The entire surfaces of two plates of seed agar (BBL) were inoculated with each strain of organism used in this experiment. After 24 hour incubation at 37°C., 4 ml sterile saline solution were added to each plate and the organisms therein suspended. About 8 ml of each strain were harvested and placed in a centrifuge tube. The cells were centrifuged down in an angle head centrifuge and had a final volume of about ¼ ml. The residue was transferred to a cooled sterile mortar. Six-tenths g of Alumina A-303 were added. The cells were ground with a pestle for 1 minute following the procedure described by McIlwain (45). The ground cells were resuspended in 2 ml sterile saline solution and 1 ml erythromycin standard solution. The suspensions were refrigerated at 6°C. for 24 hours and then left at room temperature for 24 hours. Next they were diluted with 27 ml distilled water each and were sterilized by filtration through Seitz filters. The filtrates were added to hydrolyzed casein medium in amounts equivalent to 0, 0.1, 0.4, 1.2, 3.7 and 11 mcg of erythromycin originally present per ml of final volume. All tubes were inoculated with the sensitive strain no. 3055 of *M. pyogenes* var. *aureus*. After incubation at 37°C. for 3 days, growth was determined by visual inspection.

The Effect of Erythromycin on the Action of Coagulase and Other Enzymes

Coagulase tests were carried out in a manner similar to that described in the Difco Manual (46). Plasma was prepared from rabbit blood to which 1 per cent Na-oxalate was added. Dilutions of erythromycin were made in distilled water with erythromycin standard solution. Derivatives of erythromycin were dissolved or suspended in distilled water. *Micrococcus pyogenes* var. *aureus* was grown in Brain Heart Infusion (Difco) at 37°C. for 24 hours. Suspensions of the organisms were standardized at 42.5 per cent light transmission at a wave length of 550 lambdas in a spectrophotometer. Five-tenths ml plasma were measured into an 11 mm by 65 mm culture tube; 0.1 ml erythromycin solution, derivative solution or water was added; 0.1 ml standardized suspension of *M. pyogenes* var. *aureus* cells was added last. Each tube was mixed by shaking and placed in a 37°C. water bath. Tubes were examined for coagulation every 5 minutes for the first 30 minutes and every 10 minutes up to 3 hours. The amount of coagulation was estimated by the apparent viscosity of the plasma. A numerical value of 4 was assigned when the tube could be inverted without displacement of the plasma from the bottom of the tube; a value of 0 was assigned when there was no apparent change in viscosity.

A preliminary study was made on the effect of the addition of erythromycin or its derivatives at the time of growth on the formation of coagulase by *M. pyogenes* var. *aureus*. Tubes containing 5 ml casamino acid medium to which had been added 12.5, 5 and 1.25 mcg erythromycin, 5 mg desosamine, 5 mg erythralosamine, 5 mg dihydroerythronolide, $C_{21}H_{41}O_8$, and a control were inoculated with strain no. 3055. A control medium and a medium with 100 mcg erythromycin added were inoculated with strain no. 3066. The coagulase action of these cultures was noted for the first 60 minutes. The effect of the addition of 100 mcg erythromycin at the time of the coagulase test on the coagulase activity of strain no. 3055 was also determined.

A second series of tests was carried out using cultures of sensitive strains no. 3055 and no. 3067 and resistant strains no. 3066 and no. 3074 grown in brain heart infusion alone and resistant strains no. 3066 and no. 3074 grown in the presence of 100 mcg erythromycin per ml. The coagulase activity was determined for each of these six cultures alone with the addition of 100 mcg erythromycin at the time of test.

A third series of tests was carried out using cultures of strains no. 3055, no. 3066, no. 3067 and no. 3074 grown in brain heart infusion. At the time of test, 0, 0.1, 1, 10 and 100 mcg erythromycin were added to plasma tubes with each strain of *M. pyogenes* var. *aureus* to determine the effect on coagulase activity of erythromycin.

A fourth series of tests was carried out to determine the effect of several degradation products of erythromycin on the coagulase activity of *M. pyogenes* var. *aureus*. At the time of test 1000 mcg desosamine, 1000 mcg cladenose, 100 mcg dihydroerythronolide, 100 mcg erythralosamine, and 1 mcg erythromycin were added in 0.1 ml distilled water to 0.5 oxalated plasma. One-tenth ml of suspended cells of *M. pyogenes* was added to each tube as well as to a control. Strains no. 3055, no. 3066, no. 3067 and no. 3074 were used. The time of coagulation of each tube was noted.

The new clinical aid, "Tes-Tape," depends on the activity of glucose oxidase and horse-radish peroxidase to determine the presence of glucose in the urine. A 40 mm strip of "Tes-Tape" was wet with 0.05 ml erythromycin standard solution and allowed to air dry. The antibiotic treated strip and a control strip were both wet with a 1 per cent glucose solution. Both strips were dried in the air for 1 minute. The depth of the color of each strip was compared with the color chart on the tape dispenser.

RESULTS

Screening of Compounds to Determine Reversal of Potentiation of Action of Erythromycin

Compounds Screened. Several of the compounds screened showed some form of activity. Some of these substances reversed the inhibition of the test

organisms by erythromycin; other potentiated the action of the antibiotic; still others acted independently of the erythromycin in a toxic effect on the test organisms or reacted with the media to form opaque areas.

The following compounds reversed the inhibition of test organisms by erythromycin. The diameters of the growth zones on plates seeded with *Micrococcus pyogenes* var. *aureus* and spores of *Bacillus subtilis* are here listed:

adenylic acid, 1 per cent: *B. s.*, 14 to 19 mm
ascorbic acid, 1 per cent: *M. p.*, 14 mm; *B. s.*, 14 to 18 mm
DL-aspartic acid, 2 per cent: *M. p.*, 15 to 24 mm; *B. s.*, 19 to 26 mm
citric acid, 1 per cent: *M. p.*, 22 mm; *B. s.*, 18 to 27 mm
L-cysteine-HCl, 10 per cent: *M. p.*, 23 to 33 mm; *B. s.*, 27 to 34 mm
L-glutamic acid, 1 per cent: *B. s.*, 15 to 21 mm glutathione, 1 per cent: *M. p.*, 19mm
L-histidine-HCl, 5 per cent: *M. p.*, 20 mm; *B. s.*, 22 mm
oxalic acid, 1 per cent: *M. p.*, 23 mm; *B. s.*, 27 to 33 mm

The inhibition of *Micrococcus pyogenes* var. *aureus* and spores of *Bacillus subtilis by* erythromycin was potentiated by adenine sulfate, 1 per cent, guanine-HCl, 0.7 per cent, uracil, 1 per cent, and xanthine, 1 per cent.

A number of the compounds which reversed the inhibition of erythromycin had a halo-like growth zone. In fact, the only one of these reversing compounds for which this halo-like effect was not seen was glutathione. Data giving the diameter of the inner zone of inhibition is given in the paragraph 2, page 79. In seeded plates to which erythromycin had not been added, these compounds produced zones of inhibition of about the same diameter as the inner diameter of the halo in plates to which erythromycin had been added.

Oxalic acid reacted with both media to form opaque zones around the paper disks.

Toxic effects of *Micrococcus pyogenes* var. *aureus* and spores of *Bacillus subtilis* produced inhibition zones with diameters as follows:

$FeSO_4.7H_2O$, 10 per cent: *M. p.*, 25 mm; *B. s.*, 27 mm
D-glucosamine, 1 per cent: *M. p.*, 25 mm; *B. s.*, 13mm
mercaptoethyl amine, 10 per cent: *B. s.*, 50 mm (no sharp threshold)
thioglycerol, 10 per cent: *M. p.*, 21 mm; *B. s.*, 16 mm
tetra sodium salt of ethylene diamine tetra acetic acid, 9.4 per cent: *M. p.*, 27 mm; *B. s.*, 34 mm

$FeSO_4.7H_2O$, 10 per cent, and D-glucosamine, 1 per cent, enhanced the growth of spores of *Bacillus subtilis* with zones 39 mm and 22 mm in diameter, respectively.

Hydrogen Ion Concentration. The hydrogen ion concentration was determined on solutions of all compounds screened except Ca-panthothenate, pyridoxine, niacin, biotin, desosamine, $ZnSO_4.7H_2O$, $TiSO_4$ and H_3BO_3. The hydrogen ion concentrations of most solutions were found to lie within the pH range of 5.0 to 7.9. Solutions which had a hydrogen ion concentration above or below this range had pH's as follows:

adenine sulfate, 1 per cent	10.0
adenylic acid, 1 per cent	2.7
ascorbic acid, 1 per cent	2.5
DL-aspartic acid, 2 per cent	2.9
betaine, 1 per cent	4.1
citric acid, 1 per cent	2.2
L-cysteine-HCl, 1 per cent	1.7
L-glutamic acid, 1 per cent	2.9
glutathione, 1 per cent	3.0
guanine HCl, 0.7 per cent	10.4
L-histidine-HCl, 1 per cent	4.0
mercaptoethyl amine, 10 per cent	4.6
oxalic acid, 1 per cent	3.7
p-aminobenzoic acid, 1 per cent	3.7
thiamine-HCl, 0.1 per cent	3.7
uracil, 1 per cent	9.9
xanthine, 1 per cent	10.4

The neutralized solutions of adenylic acid (4 per cent), ascorbic acid (10 per cent), L-aspartic acid (5 per cent), L-cysteine-HCl (10 per cent), L-glutamic acid (4 per cent), glutathione (2 per cent) and L-histidine-HCl (5 per cent) gave no evidence of reversing the bacteriostasis of the test organisms, *Micrococcus pyogenes* var. *aureus* and *Bacillus subtilis*, caused by threshold amounts of erythromycin.

L-Cysteine-HCl. Erythromycin which had been treated with L-cysteine-HCl at a pH of 5.2 at 56°C. for 1 hour gave zones of inhibition with both *Micrococcus pyogenes* var. *aureus* and the spores of *Bacillus subtilis* identical with those obtained from an erythromycin control which had been treated with no L-cysteine-HCl. After a subsequent 3-day storage in the refrigerator, potencies were still identical.

Organic and Inorganic Acids. It was found that the inhibition of *Micrococcus pyogenes* var. *aureus* and the spores *Bacillus subtilis* was reversed by a number of organic and mineral acids. Alanine (2 per cent) and HCl (1 per cent), glycine (2 per cent) and HCl (1 per cent), HCl (1 per cent), lactic acid (1 per cent), malic acid (1 per cent), H_2SO_4 (1 per cent) and tartaric acid (1 per cent) were effective. In the case of *B. subtilis* these acids in the aforementioned concentrations caused halo-like zones of growth in plates containing threshold amounts of the antibiotic. In the case of *M. pyogenes* the zone of growth extended to the paper disk on which the acid had been added.

Revival. It was found that revival of *Micrococcus pyogenes* var. *aureus* and spores of *Bacillus subtilis* by a threshold amount of erythromycin could be effected by the compounds which had shown reversal action when added at the same time as the antibiotic. DL-asparic acid, L-cysteine-HCl and L-histidine-HCl brought about the revival of both organisms. Growth could be seen at 24 hours but was much more evident at 48 hours. Only a small percentage of the inhibited organisms were revived. In the case of *M. pyogenes* var.

aureus most of these appeared to be growing on the surface of the agar. After becoming aware of the importance of the pH of the compounds screened, it was found that alanine (2 per cent) and HCl (1 per cent), ascorbic acid (4 per cent), L-glutamic acid (2 per cent), HCl (1 per cent) and H_2SO_4 (1 per cent) brought about the revival of *B. subtilis*. The revival of *M. pyogenes* var. *aureus* was brought about by alanine (2 per cent) and HCl (1 per cent), ascorbic acid (4 per cent), L-glutamic acid (2 per cent), malic acid (1 per cent) and tartaric acid (1 per cent).

Investigation of the Reversal by Calcium Pantothenate and Beta-Alanine of the Inhibitory Effects of Erythromycin on Several Species of Bacteria

Lactobacillus casei. In the studies with *L. casei* it was found that 0.2 mcg/ml erythromycin caused an increase in the pH of the blank tubes to which no Ca-pantothenate had been added after the tubes had been inoculated and incubated 3 days at 37°C. One mcg/ml of the antibiotic resulted in a still higher pH (less acid production). The minimal inhibitory level of erythromycin, i.e., 0.2 mcg/ml erythromycin, 0.02, 0.2 and 2 mcg/ml Ca-pantothenate caused a reversal of the inhibitory effect and brought about growth. However, when 1 mcg/ml of the antibiotic was present even 20 mcg/ml of the vitamin caused no appreciable reversal of inhibition (Table 1). When 0.2, 2 or 20 mcg/ml of erythromycin was present, up to 200 mcg/ml beta-alanine had no growth effect in the presence of 0.2 mcg/ml Ca-pantothenate (Table 2).

Saccharomyces cerevisiae. With the "Gebrüder Mayer" strain of *S. cerevisiae* 10 mcg/ml of erythromycin caused no inhibition. When 100 mcg/ml of the antibiotic was present, it took 0.02, 0.04 and 0.05 mcg/ml Ca-pantothenate to bring the growth of the yeast to the level, as measured by the spectrophotometer, which 0.005, 0.02 and 0.03 mcg/ml Ca-pantothenate attained in the absence of erythromycin (Table 3). Similarly, it required 0.4 and 0.8 mcg/ml beta-alanine in the presence of 100 mcg/ml erythromycin to bring about growth equivalent to that of 0.2 an 0.4 mcg/ml beta-alanine in the absence of erythromycin (Table 4).

It was necessary to use the level of 100 mcg/ml erythromycin to cause a reduction of growth of the yeast. It was impractical to use higher concentrations of the antibiotic due to its limited solubility.

Micrococcus pyogenes var. *aureus.* Strain no. 3055 of *M. pyogenes* var. *aureus* was inhibited by 0.5 mcg/ml erythromycin in the absence of either Ca-pantothenate or beta-alanine; strain no. 3067 was inhibited by 1.0 mcg/ml. With 0.1, 1 and 10 mcg/ml Ca-pantothenate added to the medium, 1.0, 0.5 and 0.5 mcg/ml of the antibiotic were required for inhibition of strain no. 3055 and 1.0, 1.0 and 1.0 mcg/ml of the antibiotic for strain no.

3067. With 10, 100 and 1000 mcg/ml beta-alanine added, 0.5, 0.5 and 0.5 mcg/ml erythromycin were required for inhibition of strain no. 3055 and 1.0, 1.0 and 2.0 mcg/ml, for strain no. 3067. Neither Ca-pantothenate or beta-alanine reversed the inhibition of these strains by erythromycin appreciably (Table 5).

Diplococcus pneumoniae. The growth of *D. pneumoniae* was partially inhibited by 0.005 mcg/ml erythromycin and completely inhibited by 0.01 mcg/ml in the absence of Ca-pantothenate or beta-alanine. The presence of 0.1, 1 and 10 mcg/ml Ca-pantothenate, the organism was inhibited by 0.01 mcg/ml of the antibiotic at all levels. In the presence of 10, 100 and 1000 mcg/ml beta-alanine, the organism was inhibited by 0.01 mcg/ml erythromycin at all levels. Neither growth factor reversed the inhibition of the organism by the antibiotic (Table 6).

Streptococcus pyogenes. The growth of *S. pyogenes* was partially inhibited by 0.005 mcg/ml and completely inhibited by 0.01 or 0.02 erythromycin in the absence of Ca-pantothenate or beta-alanine. In the presence of 0.1, 1 and 10 mcg/ml Ca-pantothenate, the organism was inhibited at all levels by 0.02 mcg/ml of the antibiotic. In the presence of 10, 100 and 1000 mcg/ml beta-alanine, the organism was inhibited by 0.01, 0.02 and 0.02 mcg/ml of the antibiotic, respectively. Neither Ca-pantothenate nor beta-alanine caused any appreciable reversal of the inhibition of *S. pyogenes* by erythromycin (Table 6).

Corynebacterium diphtheriae. The inhibition of *C. diphtheriae* by erythromycin showed no sharp cut-off point. A 0.004 mcg/ml concentration of the antibiotic permitted maximum growth of the organism; 0.008 mcg/ml, permitted heavy growth; 0.016 mcg/ml, permitted moderate growth; and 0.032 mcg/ml, permitted slight growth. In the presence of 0.1 and 1 mcg/ml Ca-pantothenate or 10, 100 and 1000 mcg/ml beta-alanine, the different concentrations of erythromycin produced the same pattern of inhibition as in the control which received neither nutrient. When 10 mcg/ml Ca-pantothenate was used, the partial inhibition caused by 0.008 and 0.016 mcg/ml erythromycin appeared to be reversed and that caused by 0.032 mcg/ml of the antibiotic was not affected (Table 7).

Gots' Method. Using the two sensitive strains of *Micrococcus pyogenes* var. *aureus*, no. 3055 and no. 3067, in seeded plates, there was no evidence that the resistant strains of *M. pyogenes* var. *aureus*, no. 3066 and no. 3074, or the resistant organisms *Proteus vulgaris* and *Pseudomonas aeruginosa* produced a metabolite which was capable of diffusing through the agar and reversing the inhibition of erythromycin.

Production of Pantothenic Acid by Resistant Strains of Micrococcus pyogenes var. aureus. Mylase-treated, autoclaved suspensions of resistant and sensitive strains of *M. pyogenes* were assayed for pantothentic acid content. The suspension of sensitive strain no. 3055 contained 0.51 mcg/ml panto-

thenic acid; resistant strain no. 3066 contained 0.44 mcg/ml; resistant strain no. 3074 contained 0.43 mcg/ml.

Investigation of the Presence of Intracellular and Extracellular Erythromycinase

Gots' Method, Extracellular Erythromycinase. Using the method of Gots (4), agar seeded with sensitive strains no. 3055 and no. 3067 of *Micrococcus pyogenes* var. *aureus* gave no indication of a diffusible extracellular erythromycinase in either resistant strains no. 3066 and no. 3074 of *M. pyogenes* var. *aureus* or in the resistant organisms *Proteus vulgaris* and *Pseudomonas aeruginosa.* Using bacteriostatic amounts of the antibiotic in the agar, there was no growth of the sensitive seeded organisms in the proximity of the resistant organisms.

Breakdown of Erythromycin in Solution by Extracellular Erythromycinase. After incubation the erythromycin containing media surrounding the dialyzing tubes which were inoculated with resistant strains no. 3066 and no. 3074 of *Micrococcus pyogenes* var. *aureus* and the antibiotic control still inhibited the growth of the sensitive strain no. 3055 at dilutions up through 1:64. The second control, which contained no erythromycin and had been inoculated with sensitive strain no. 3055, produced no substance capable of reducing growth (Table 8).

Solvent Treatment of Cells to Release Intracellular Erythromycinase. Erythromycin solutions which had been treated with the solvent-extracted, resistant strains of *Micrococcus pyogenes* var. *aureus* had identical inhibitory powers. In each case after treatment by defatted cells of strains no. 3066 and no. 3074, dilutions equivalent to an original concentration of 0.39 mcg/ml erythromycin inhibited the growth of sensitive strain no. 3055 of *M. pyogenes* var. *aureus;* dilutions equivalent to an original concentration of 0.10 mcg/ml did not inhibit the growth (Table 9).

Physical Destruction of Cell Wall to Release Intracellular Erythromycinase. Dilutions of erythromycin which had been treated with alumina ground cells of resistant strains no. 3066 and no. 3074 of *Micrococcus pyogenes* var. *aureus* and the resistant organisms *Proteus vulgaris* and *Pseudomonas aeruginosa* and had been subsequently sterilized retained much erythromycin activity. In all four cases dilutions equivalent to 1.2 mcg/ml original erythromycin content inhibited growth of the sensitive strain no. 3055 of *M. pyogenes* var. *aureus;* dilutions equivalent to 0.4 mcg/ml original erythromycin content did not inhibit growth (Table 10).

The Effect of Erythromycin on the Action of Coagulase and other Enzymes

In the preliminary test the presence of coagulase was varied when erythromycin and several erythromycin degradation compounds had been added to

the growing organism *Micrococcus pyogenes* var. *aureus*. Using strain no. 3055, the control with no compound added, cultures grown with 1 mg/ml desosamine, 1 mg/ml erythralosamine and 1 mg/ml dihydroerythronolide all gave a coagulation value of 4 after 60 minutes. The addition of 100 mcg erythromycin at the time of the test reduced the coagulation value of no. 3055 to 3 at 100 minutes. Using strain no. 3066, the control gave a coagulation value of 3 at 60 minutes and the addition of 100 mcg erythromycin at the time of test reduced the coagulation value to 1 at 60 minutes.

In the second series of tests, using suspensions of *M. pyogenes* var. *aureus* which had been standardized by a spectrophotometer, the addition of 100 mcg erythromycin to the mixture of plasma and suspension of organisms at the time of test increased the time required for coagulation. The six suspensions of the organism coagulated the plasma in periods of time varying from 40 to 80 minutes. When 100 mcg erythromycin was added at the time of the test, the time required for coagulation was 80 minutes to greater than 3 hours. The increase in time to bring about coagulation due to the addition of erythromycin at the time of the test varied from 40 minutes to greater than 140 minutes (Table 11).

A third test, with all four strains of *M. pyogenes* var. *aureus*, revealed that amounts of erythromycin as small as 0.1 mcg increased the time of coagulation. With all four strains the addition of 1 mcg erythromycin resulted in still further increases of time required for coagulation. Amounts of erythromycin in excess of 1 mcg, namely 10 and 100 mcg, did not further increase time required for coagulation under test conditions but took longer for coagulation than those tubes which received 0 or 0.1 mcg of the antibiotic (Table 12).

It was found that the addition of 100 mcg/ml erythromycin increased the growth for resistant strains no. 3066 and no. 3074 of *M. pyogenes* var. *aureus* as measured in percentage light transmission from 23 to 20 and 34 to 28, respectively.

In a final test, the effect of several degradation products of erythromycin on the time required for coagulation was found to vary among the same four strains of *M. pyogenes* var. *aureus*. The average time required for formation of a coagulum, firm enough to allow inverting the tube without loss of the plasma, was 82 minutes. The addition of 1000 mcg desosamine required on the average 76 minutes; 1000 mcg cladenose, 80 minutes; 100 mcg erythralosamine, 97 minutes; 100 mcg dihydroerythronolide, 112 minutes; and 1 mcg erythromycin, over 160 minutes (Table 13).

"Tes-Tape," the glucose oxidase and horse-radish peroxidase impregnated paper, was not affected in its reaction to glucose by erythromycin. Tape previously saturated with erythromycin standard solution reacted to give the same color upon being wet by glucose solution as a control.

DISCUSSION

Screening of Compounds to Determine Reversal of Potentiation of the Action of Erythromycin

Nearly one hundred compounds were screend for effect on the inhibition of growth of *Micrococcus pyogenes* var. *aureus* and the spores of *Bacillus subtilis* by erythromycin. Solutions of four of these compounds potentiated the action of the antibiotic and solutions of nine of the compounds reversed the action. The four compounds which had a potentiating effect were purines and had been dissolved, using NaOH, with resulting pH's of 9.9 to 10.4. Solutions of the nine compounds which had shown reversal of the action of erythromycin were all acidic with pH's ranging from 1.7 to 4.0. Since the work of Pittenger et al. (1) and Haight and Finland (2) indicated that an increase in pH resulted in increased activity of erythromycin, it appeared that both the potentiating and reversing phenomena resulted from the hydrogen ion concentration of the solutions screened.

The neutralization of the solutions of screened compounds which showed activity afforded an opportunity to determine whether the structure alone could account for activity. With the four purines, adenine, guanine, uracil and xanthine, neutralization would result in their precipitation and, therefore, no useful data could be obtained. Solutions of seven of the nine active acidic compounds were brought to pH's between 6.2 and 7.0. None of these solutions retained the ability after neutralization to reverse the action of erythromycin. As a complementary measure, various other organic and inorganic acids were found to reverse the action of the antibiotic.

Revival, apparently, presents a similar situation. Only solutions of acid compounds brought about revival and they were for the most part the same as those which caused reversal. It would appear that the greater proportion of the *Micrococcus pyogenes* var. *aureus* and *Bacillus subtilis* cells had been killed by the "bacteriostatic" concentration of erythromycin. The remaining resistant bacteria resumed growth after the shift to the acid side, by the addition of the solution to the paper disk, reduced the effectiveness of the antibiotic to below the bacteriostatic level.

The halo phenomenon was exhibited in both reversal and revival. In plates seeded with the spores of *Bacillus subtilis* the diameters of the inner zone of inhibition were larger than those of plates seeded with *Micrococcus pyogenes* var. *aureus*. The inner zone of inhibition adjacent to the paper disk appears to be a toxic effect of a low pH. Since *Bacillus subtilis* requires more alkaline surroundings it follows that the zone would necessarily be larger. With an increase in distance from the paper disk one would expect the pH to rise to a level where the bacteria were not inhibited and at which the concentration of antibiotic present would be reversed and, finally, at a still

greater distance the strength of the acid would have been so reduced that the organisms would again be inhibited by the antibiotic.

It had been suspected that the sulfhydryl radical of cysteine or the closely related mercaptoethylamine might react with a ketone group in erythromycin but there appears to be no reaction.

Desosamine, the nitrogen containing sugar which is found in numerous antibiotics of the erythromycin group, exhibited no activity of any kind. The activity of erythromycin is due to other characteristics of the molecule.

Investigation of the Reversal by Calcium Pantothenate and Beta-Alanine of the Inhibitory Effects of Erythromycin on Several Species of Bacteria

Very little has been found to support the thesis that Ca-pantothenate and beta-alanine act as antagonists of erythromycin. Brown and Emerson (4) used Ca-pantothenate and beta-alanine to reverse the inhibition of *Corynebacterium diphtheriae* by erythromycin. Employing the same medium but using different strains of the bacterium, Ca-pantothenate and beta-alanine did not reverse the action of erythromycin. In this study ratios of Ca-pantothenate and beta-alanine to the antibiotic equalled or surpassed those used by Brown and Emerson. The data reported here do not confirm their findings, although other strains were used.

Working with other organisms and various technics, indications of the reversal of the action of erythromycin by Ca-pantothenate or beta-alanine were found in two instances. The "Gebrüder Mayer" strain of *Saccharomyces cerevisiae* showed reversal of inhibition due to the antibiotic in the presence of both Ca-pantothenate and beta-alanine. The reversal of erythromycin was seen at only one inhibitory concentration of the antibiotic since higher levels were not practical due to its limited solubility. Therefore the nature of the reversal cannot be determined. Two alternative explanations of the results can be made. Since the yeast requires either Ca-pantothenate or beta-alanine, the antibiotic cannot be inhibiting the growth and division by blocking the production of Ca-pantothenate or beta-alanine. It is possible, however, that the erythromycin acts by competing with Ca-pantothenate or beta-alanine for a position in an essential enzyme system. As an alternative, erythromycin could be interfering with an essential enzyme system which is distinct from the enzyme system in which Ca-pantothenate and beta-alanine play an important role. In this situation the addition of a sub-bacteriostatic amount of erythromycin and the presence of a low amount of Ca-pantothenate would interfere with the normal operation of the respective enzyme systems. While the reduced action of either enzyme system alone would not prevent growth and reproduction, the reduced action of both enzyme systems would, synergistically, inhibit growth or reproduction. If the reversal is competitive, it may be estimated that either 0.02 mcg Ca-pantothenate or

1 mcg beta-alanine reverse the action of 100 mcg erythromycin. The activity coefficients of Ca-pantothenate and beta-alanine would be 5,000 and 100, respectively. *Lactobacillus casei* showed no reversal of the action of the antibiotic with beta-alanine; but in the presence of the minimal inhibiting concentration of erythromycin, the bacterium showed an apparently non-competitive reversal of the inhibition by Ca-pantothenate.

In the other organisms studied, Ca-pantothenate and beta-alanine did not reverse the inhibitory action of erythromycin. The inhibition by erythromycin of *Diplococcus pneumoniae, Micrococcus pyogenes* var. *aureus,* and *Streptococcus pyogenes* was not reversed by Ca-pantothenate at concentrations as high as 10 mcg/ml or beta-alanine at concentrations as high as 1000 mcg/ml. The average level of pantothenic acid in human blood was found by Pearson (47) to be 0.19 mcg/ml. It appears unlikely that vitamin therapy would increase the pantothenic acid above blood levels at which no reversal of the action of erythromycin would occur.

If the action of erythromycin were reversed by Ca-pantothenate, resistance of organisms might be due to synthesis of large amounts of that vitamin. Amounts of pantothenic acid produced by two highly resistant strains of *Micrococcus pyogenes* var. *aureus* were almost equal to that produced by a sensitive strain. The enzyme mylase made pantothenic acid in a bound form available for assay. These data are convincing in showing the lack of any competitive relationship between erythromycin and pantothenic acid. Since beta-alanine is a precursor of pantothenic acid (48), the above data constitute indirect evidence that synthesis of beta-alanine is not the means by which these strains are resistant to the antibiotic.

The evidence that the resistant strains of *Micrococcus pyogenes* var. *aureus* and the two organisms, *Proteus vulgaris* and *Pseudomonas aeruginosa,* do not produce extracellular erythromycinase, is equally valid as evidence that they do not produce extracellular metabolites competitive with erythromycin.

With the possible exception of data found using the "Gebrüder Mayer" strain of *Saccharomyces cerevisiae,* all results indicated that neither Ca-pantothenate or beta-alanine competitively reversed inhibition caused by erythromycin.

Investigation of the Presence of Intracellular and Extracellular Erythromycinase

One mechanism which might account for the resistance of certain strains and organisms to erythromycin would be the presence of erythromycinase. The enzyme might be present either in an extracellular form or in an intracellular form.

Neither of the two resistant *Micrococcus pyogenes* var. *aureus* strains nor *Proteus vulgaris* nor *Pseudomonas aeruginosa* reversed the inhibition of the

two sensitive seed organisms by erythromycin. In addition, resistant strains of *M. pyogenes* var. *aureus* were grown in dialyzing tubes surrounded by media containing erythromycin. It should be expected that the erythromycin would diffuse through the cellophane tube to the organisms and be broken down if erythromycinase were present. Here again there was no indication of destruction.

It should be noted that if apparent destruction of the antibiotic had occurred it would be necessary to determine whether erythromycinase had destroyed the erythromycin or a competitive metabolite had been produced.

Since no extracellular erythromycinase was found, it can be concluded that the resistant strains of *Micrococcus pyogenes* var. *aureus* are truly resistant. It had been found previously that some "resistant" organisms produce an antibiotic destroying enzyme which changes the environment by reducing the antibiotic concentration below the threshold level so that the truly sensitive organism can grow. Such is not the case here.

The possibility of an intracellular erythromycinase still remained. The treatment of cells by fat solvents has been found to render the walls of the cells permeable enabling molecules as large as penicillin, for example, to come in contact with enzymes in cells (4). There was no evidence, however, of erythromycinase in the solvent-treated strains studied. The use of high frequency sound waves to break down the cell walls was rejected because the cells of *Micrococcus pyogenes* var. *aureus* offer great resistance to this treatment. Confirmation by grinding with alundum was chosen since it is effective and yet causes a minimum of denaturation of the protein of the cell. Apparently there was some absorption of the antibiotic on the alundum and the contents of the cells; but the amount of erythromycin left free in the solution after treatment was still many times that necessary to inhibit the growth of a sensitive strain. Thus, the evidence is strong that intracellular erythromycinase is not present in the two resistant strains of *M. pyogenes* var. *aureus* and in *Proteus vulgaris* and *Pseudomonas aeruginosa*.

No evidence of either extracellular or intracellular erythromycinase was found in the organisms studied.

The Effect of Erythromycin on the Action of Coagulase and Other Enzymes

Originally it had been suspected that the effectiveness of erythromycin in vivo in mice against strains of *Micrococcus pyogenes* var. *aureus* was due to an effect on coagulase by erythromycin. This matter was pursued when Boniece et al. (34) revealed that erythromycin in small amounts reduced the activity of coagulase. From the results obtained using four strains of *Micrococcus pyogenes* var. *aureus* there is no doubt as to the reduction of the speed of coagulase activity when small amounts of erythromycin were added at the time of test. Furthermore, the degradation compounds, cladenose, desosa-

mine, dihydroerythronolide, and erythralosamine, had little if any activity compared with erythromycin. It appears that the property of reducing the activity of coagulase is evanescent as the antibiotic is cleaved into its component parts. The data concerning dihydroerythronolide and erythralosamine was not statistically significant and it would be worth while to make further tests of the effect of these compounds on coagulase. The inhibitory action of erythromycin cannot be associated with its degradation compounds, desosamine, erythralosamine, and dihydroerythronolide.

One conclusion is obvious, erythromycin interferes with sensitive strains of *Micrococcus pyogenes* var. *aureus* in at least two ways: it inhibits portions of the metabolic system which function in the growth and reproduction of the organism; and it interferes with the action of coagulase. It should be noted, however, that coagulase was still formed by resistant strains growing in the presence of the antibiotic. Erythromycin interferes with the action of coagulase not with its production.

It would appear that with the finding of an antibiotic which protects mice in vivo against in vitro resistant *Micrococcus pyogenes* var. *aureus,* and the fact that the antibiotic reduces coagulase activity, one may have found a phenomenon of considerable utility. Let it be assumed that erythromycin counteracts *M. pyogenes* var. *aureus* in vivo by reducing the speed of coagulase activity. Since the antibiotic does not inhibit the reproduction of the organism, but inhibits the action of one of its enzymes acting externally, it would appear that there would be little tendency for the development of an in-vivo-fast strain which would produce a coagulase more resistant to the action of erythromycin. Since this is a most versatile organism, it would appear more likely that the protection afforded to the animal by the use of the antibiotic would favor the development of strains which might be resistant to other natural host defenses and might kill the host by a method in no way connected with the coagulase action.

The effect on the speed of coagulase activity may be an in vitro index for screening drugs and antibiotics for in vivo effectiveness against *Micrococcus pyogenes* var. *aureus*. The finding of active substances by this in vitro method which would be also active in vivo, would, aside from the possibility of clinical usefulness, throw additional light on the manner in which *M. pyogenes* var. *aureus* behaves in vivo.

CONCLUSION

Erythromycin is antibiotic by its interference with the normal functioning of one or more enzyme systems in susceptible organisms.

A screening of nearly one hundred compounds, using *Micrococcus pyogenes* var. *aureus* and the spores of *Bacillus subtilis,* revealed no substances capable of reversing the inhibition caused by erythromycin. Compounds in

acid solution acted to reverse the activity of erythromycin and compounds in alkaline solution acted to potentiate its activity; but the basic cause was the acidity or alkalinity of the solution.

For clinical purposes, the revival of organisms inhibited by erythromycin would appear to be favored by planting into media with as low a pH as would be compatible with the organism.

No evidence was found that Ca-pantothenate or beta-alanine reversed competitively the inhibition by erythromycin of *Corynebacterium diphtheriae*, *Diplococcus pneumoniae*, *Lactobacillus casei*, *Micrococcus pyogenes* var. *aureus* and *Streptococcus pyogenes*.

Both Ca-pantothenate and beta-alanine brought about reversal of the inhibition of *Saccharomyces cerevisiae* by erythromycin. Owing to solubility limitations it was not possible to use larger amounts of the antibiotic and determine the presence of a competitive reversal of the type exhibited by p-aminobenzoic acid and the sulfa drugs over large variations of concentration.

No evidence was found of the synthesis of a metabolite competitive with erythromycin by resistant organisms. Resistant strains of *Micrococcus pyogenes* var. *aureus* synthesized the same amount of pantothenic acid as a sensitive strain. Gots' test demonstrated that resistant strains of *M. pyogenes* var. *aureus*, *Proteus vulgaris*, and *Pseudomonas aeruginosa* synthesized no extracellular metabolite capable of reversing the action of erythromycin.

Neither extracellular nor intracellular erythromycinase was found in *Micrococcus pyogenes* var. *aureus*, *Proteus vulgaris*, or *Pseudomonas aeruginosa*. Neither solvent treatment nor mechanical rupturing of cells released such an enzyme.

Desosamine, a structure common to numerous members of the erythromycin antibiotic group, has no effect on the action of erythromycin and has no bacteriostatic effect on *Micrococcus pyogenes* var. *aureus* or on the spores of *Bacillus subtilis*.

The finding of Boniece et al. (34) that erythromycin reduced the activity of the coagulase of *Micrococcus pyogenes* var. *aureus* was confirmed. Desosamine and cladenose, degradation products of erythromycin, did not reduce the activity of coagulase. It is suggested that additional work should be done to determine whether the reduction of activity of coagulase by dihydroerythronolide and erythralosamine is significant. None of the four degradation products of erythromycin tested had an effect on coagulase which approached that of the antibiotic.

Erythromycin had no effect on the enzymes glucose oxidase and horseradish peroxidase.

It is suggested that in view of erythromycin's reduction of the activity of coagulase, there may be little tendency for "in vivo resistant" strains of *Micrococcus pyogenes* var. *aureus* to develop. The coagulase activity test is

recommended as an in vitro method for the testing of drugs for their in vivo effectiveness against *M. pyogenes* var. *aureus*.

The inactivity of various metabolites on the action of erythromycin, the inability of Ca-pantothenate and beta-alanine to reverse the inhibition of numerous organisms by the antibiotic, and the apparent absence of erythromycinase, lead to the conjecture that the most fruitful areas of study of the mode of action would be: 1. the investigation of the effect of erythromycin on the various enzyme systems in the bacterial cell; 2. the comparison of the enzyme systems of sensitive and resistant strains of the same organism.

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TABLE 1

Effect of Calcium Pantothenate and Erythromycin on the Hydrogen Ion Concentration of *Lactobacillus casei*

	erythromycin mcg/ml			
Calcium Panto-thenate mcg/ml	0.0 pH	0.04 pH	0.2 pH	1.0 pH
0.0	5.5	5.5	5.8	6.5
0.02	4.4	4.5	5.1	6.4
0.2	4.1	4.1	4.6	6.4
2.0	4.1	4.2	4.8	6.4
20.0	4.1	4.1	4.8	6.4

TABLE 2

Effect of Beta-Alanine and Erythromycin of the Hydrogen Ion Concentration of *Lactobacillus casei*

	erythromycin mcg/ml			
Beta-Alanine mcg/ml	0.0	0.2	2.0	20.0
	pH	pH	pH	pH
0.0	4.1	4.7	6.7	6.7
0.2	4.2	4.6	6.7	6.7
2.0	4.1	4.7	6.7	6.7
20.0	4.2	4.7	6.7	6.7
200.0	4.1	4.6	6.7	6.7

TABLE 3

Effect of Calcium Pantothenate and Erythromycin on the Growth of "Gebrüder Mayer" Strain of *Saccharomyces cerevisiae*

	erythromycin mcg/ml			
Calcium Pantothenate mcg/ml	0	1	10	100
		(readings in optical density)		
0.000	.014	.016	.015	.010
0.005	.081	.073	.072	.044
0.01	.092	.095	.080	.055
0.02	.121	.116	.122	.078
0.03	.127	.122	.126	.102
0.04	.134	.128	.134	.123
0.05	.140	.136	.137	.129

TABLE 4

Effect of Beta-Alanine and Erythromycin on the Growth of "Gebrüder Mayer" Strain of *Saccharomyces cerevisiae*

Beta-Alanine mcg/ml	erythromycin mcg/ml 0	1	10	100
	(readings in optical density)			
0.0	.024	.009	.004	.003
0.2	.116	.135	—	.054
0.4	.216	.211	.195	.126
0.8	.242	.266	.264	.224
1.6	.276	.290	.274	.276
3.2	.300	.294	.296	.282

TABLE 5

Effect of Beta-Alanine and Calcium Pantothenate on the Growth* of *Micrococcus pyogenes* var. *aureus*, Strains no. 3055 and 3067.

	erythromycin mcg/ml									
	Strain no. 3055					Strain no. 3067				
Calcium Pantothenate mcg/ml	0.0	0.1	0.2	0.5	1.0	0.0	0.1	0.2	0.5	1.0
0.0	4	4	4	0	0	4	4	4	0	0
0.1	4	4	4	4	0	4	4	4	0	0
1.0	4	4	4	0	0	4	4	4	0	0
10.0	4	4	4	0	0	4	4	4	0	0
Beta Alanine mcg/ml										
0	4	4	4	0	0	4	4	4	0	0
10	4	4	4	0	0	4	4	4	0	0
100	4	4	4	0	0	4	4	4	0	0
1000	4	4	4	0	0	4	4	4	0	0

* Growth was determined by visual estimate of turbidity. Very heavy growth was given a value of 4; heavy growth, 3; moderate growth, 2; slight growth, 1; and no growth, 0.

TABLE 6

Effect of Beta-Alanine and Calcium Pantothenate on the Growth* of *Diplococcus pneumoniae* Park I and *Streptococcus pyogenes* C203

	D. pneumoniae					*S. pyogenes*				
	erythromycin mcg/ml									
	.000	.005	.010	.020	.040	.000	.005	.010	.020	.040
Ca-Pantothenate mcg/ml										
0.0	4	2	0	0	0	4	2	2	0	0
0.1	4	2	0	0	0	4	2	2	0	0
1.0	4	2	0	0	0	4	2	2	0	0
10.0	4	2	0	0	0	4	2	2	0	0
Beta-Alanine mcg/ml										
0	4	2	0	0	0	4	2	0	0	0
10	4	2	0	0	0	4	2	0	0	0
100	4	2	0	0	0	4	2	2	0	0
1000	4	2	0	0	0	4	2	2	0	0

* Growth was determined by visual estimate of turbidity. Very heavy growth was given a value of 4; heavy growth, 3; moderate growth, 2; slight growth, 1; and no growth, 0.

TABLE 7

Effect of Beta-Alanine and Calcium Pantothenate on the Growth* of *Corynebacterium diphtheriae*

	Strain X-166					Strain Toronto				
	erythromycin mcg/ml									
	.000	.004	.008	.016	.032	.000	.004	.008	.016	.032
Ca-Pantothenate mcg/ml										
0.0	4	4	3	2	1	4	4	3	2	1
0.1	4	4	3	2	1	4	4	3	2	1
1.0	4	4	3	2	1	4	4	3	2	1
10.0	4	4	4	4	1	4	4	4	4	1
Beta-Alanine mcg/ml										
0	4	4	3	2	1	4	4	3	2	1
10	4	4	3	2	1	4	4	3	2	1
100	4	4	3	2	1	4	4	3	2	1
1000	4	4	3	2	1	4	4	3	2	1

* Growth was determined by visual estimate of turbidity. Very heavy growth was given a value of 4; heavy growth, 3; moderate growth, 2; slight growth, 1; and no growth, 0.

TABLE 8

Growth* of *Micrococcus pyogenes* var. *aureus* in the Presence of Dilutions of Erythromycin Treated by Contact with Dialyzing Tubes Seeded with Resistant Strains of *M. pyogenes* var. *aureus*

	Not Inoculated	Strain no. 3066	Strain no.3074	Strain no. 3055
		erythromycin mcg/ml (original concentration)		
Dilution of medium from outside of dialyzing tubes	100	100	100	0
1 : 2	0	0	0	4
1 : 4	0	0	0	4
1 : 8	0	0	0	4
1 : 16	0	0	0	4
1 : 32	0	0	0	4
1 : 64	0	0	0	4

* Growth was determined by visual estimate of turbidity. Very heavy growth was given a value of 4; heavy growth, 3; moderate growth, 2; slight growth, 1; and no growth, 0.

TABLE 9

Growth* of *Micrococcus pyogenes* var. *aureus* in the Presence of Dilutions of Erythromycin Treated by Solvent-Extracted Resistant Strains of *M. pyogenes* var. *aureus*

Equivalent of Erythromycin Originally Present after Dilution mcg/ml	Control	Strain no. 3066	Strain no. 3074
100.00	0	0	0
25.00	0	0	0
6.25	0	0	0
1.56	0	0	0
0.39	0	0	0
0.10	4	4	4
0.00	4	4	4

* Growth was determined by visual estimate of turbidity. Very heavy growth was given a value of 4; heavy growth, 3; moderate growth, 2; slight growth, 1; and no growth, 0.

TABLE 10

Growth* of *Micrococcus pyogenes* var. *aureus* in the Presence of Dilutions of Erythromycin Treated by the Ruptured cells of Four Bacterial Strains

Equivalent of Erythromycin Originally Present after Dilution mcg/ml	*Micrococcus pyogenes* var. *aureus* Strain no. 3066	*Micrococcus pyogenes* var. *aureus* Strain no. 3074	*Proteus vulgaris*	*Pseudomonas aeruginosa*
11.0	0	0	0	0
3.7	0	0	0	0
1.2	0	0	0	0
0.4	4	4	4	4
0.13	4	4	4	4
0.0	4	4	4	4

* Growth was determined by a visual estimate of turbidity. Very heavy growth was given a value of 4; heavy growth, 3; moderate growth, 2; slight growth, 1; and no growth, 0.

TABLE 11

Effect of Erythromycin on the Coagulation of Plasma by *Micrococcus pyogenes* var. *aureus*

Strain no.	3055	3066	3066	3067	3074	3074
Erythromycin during growth mcg/ml	0	0	100	0	0	100
Erythromycin added at time of test mcg/ml	minutes required for coagulation					
0	40	40	40	50	80	50
100	80	over 180	130	140	180	180
Increase in time due to erythromycin	40	over 140	90	90	100	130

TABLE 12

Effect of Different Concentrations of Erythromycin on the Coagulation of Plasma by *Micrococcus pyogenes* var. *aureus*

Strain no.	3055	3066	3067	3074
Erythromycin added at time of test mcg/ml	minutes required for coagulation			
0	30	40	40	50
0.1	40	60	80	70
1	70	130	150	over 180
10	70	130	100	130
100	70	90	90	100

TABLE 13

Effect of Degradation Products of Erythromycin on the Coagulation of Plasma by *Micrococcus pyogenes* var. *aureus*

Strain no.	3055	3066	3067	3074
Compound added at time of test	minutes required for coagulation			
None, control	30	70	130	100
Desosamine, 1000 mcg	40	60	100	100
Cladenose, 1000 mcg	50	70	100	100
Erythralosamine, 100 mcg	60	70	130	130
Dihydroerythronolide 100 mcg	60	130	130	130
Erythromycin, 1 mcg	100	over 180	over 180	over 180

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GROWTH RESPONSES OF ALPINE *POTENTILLA DIVERSIFOLIA* AND *ACHILLEA LANULOSA* TO GIBBERELLIC ACID

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Applications of gibberellic acid have been shown to alter the growth patterns of genetic dwarfs in maize (Phinney 1956) and in peas (Brain and Hemming 1955). Since the dwarf alpine habits of some native species have been demonstrated also to be genetically conditioned, among which are included certain species of *Potentilla* and *Achillea* (Clausen, Keck, and Hiesey 1940), the present study was an attempt to investigate the possibility of growth alterations of alpine *Achillea lanulosa alpicola* (Rydb.) Keck and *Potentilla diversifolia* Lehm. when treated with gibberellic acid. Voucher specimens of these species are in the personal herbarium of the author.

METHODS

Plants of *P. diversifolia* and *A. lanulosa* were transplanted to cans, using the soil in which they were growing, on July 11, 1957, at Cumberland Pass in Gunnison County, Colorado, at an elevation of 12,015 feet and transferred to the Rocky Mountain Biological Laboratory (also in Gunnison County) at 9,500 feet. Two plants of each species were sprayed daily with a 0.01 per cent aqueous solution of gibberellic acid, while two untreated plants of each species were kept as controls. At the beginning of the experiment on July 15, 1957, measurements of total height were taken for all plants. On August 17, 1957, measurements of maximum height were again recorded, and the plants photographed at this time (Fig. 1). All the plants included in the experiment were then preserved in formalin-acetic acid-alcohol, and measurements were made on comparable leaves. A collection of additional plants of *A. lanulosa* and *P. diversifolia* was made on August 19, 1957, at the same alpine locality, in order to compare growth of undisturbed alpine plants with the transplanted material.

RESULTS

During the 34 days of the experiment, treatment with gibberellic acid increased growth of *Potentilla* by 120 per cent and *Achillea* 89 per cent over that of the controls. Although the root systems were not studied quantitatively, the treated plants bore noticeably more roots than did the controls

(Fig. 1). In *Potentilla* total length of the earlier formed leaf of the treated plant was 55.0 per cent greater than that of the control. Ninety-nine and six-tenths per cent of this increased length was the result of a longer petiole in the treated plant as compared to the control. Length of the treated subsequently formed leaf was 72.5 per cent more than that of the control. The petiole contributed 76.2 per cent of this increase. In *Achillea*, on the other hand, total length of the earlier formed leaf of the control plant was 0.02 per cent more than that of the treated leaf. Although this difference in total length is negligible, petiole and blade proportions differed, in that the petiole was 10.4 per cent more of the total leaf length in the treated plant than in the control. The subsequently formed leaf of the treated plant grew 24.6 per cent more than did the comparable leaf of the control. Of this difference 54.9 per cent was due to the greater petiole length in the treated plant than in the control.

In *Potentilla* comparable leaves of the control transplanted from the alpine to the subalpine location grew 57.5 per cent and 142.0 per cent more than those of the alpine control. These differences were the result of 47.6 per cent more petiole in the earlier formed subalpine control leaf and 87.3 per cent increased petiole in the subsequently formed leaf of the subalpine control plant as compared to the alpine control. Comparable leaves of the subalpine control plant of *Achillea* had increased lengths of 168.9 per cent and 132.2 per cent more than those of the alpine control plant. In the earlier formed leaf 21.8 per cent of this increase in the subalpine control over that of the alpine control was due to petiole increase, while in the subsequently formed leaf 36.0 per cent of the subalpine control increases resulted from petiole length greater than that in the alpine control.

DISCUSSION

Applications of gibberellic acid, then, appear to alter the growth pattern of dwarfed alpine *A. lanulosa* and *P. diversifolia*. Root growth appears to be more abundant in the treated plants as compared to the controls in both species, especially in *Achillea*. Total height as compared to the increase in the controls is also greater with the addition of gibberellic acid (Fig. 1). Most of the leaves of the treated plants are longer than those of the controls. This increased length seems mainly the result of greater petiole length in the treated plants as compared to the controls. It is of interest that transfer to the subalpine environment produced leaf length considerably greater than that of plants remaining in the alpine habitat, an effect similar to that of gibberellic acid. It can be hypothesized that environmental change accentuates expression of the growth pattern restricted by the alpine environment and that application of gibberellic acid pushes further an expression normally blocked by gene action affecting growth hormones.

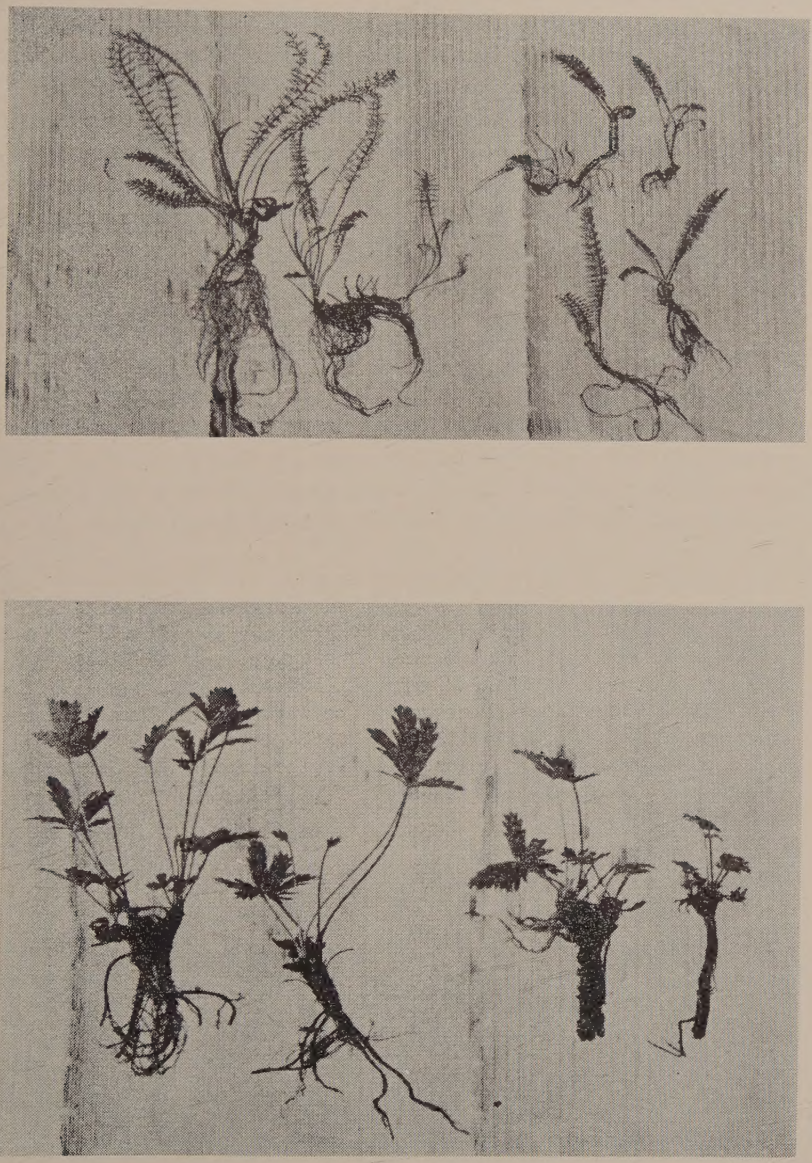

Fig. 1 (Above) *Achillea lanulosa alpicola* (Below) *Potentilla diversifolia*. Plants at the left in each species have been sprayed daily with a 0.01 per cent aqueous solution of gibberellic acid for 34 days, while those at the right in each case are the untreated controls. The two control plants of *Achillea* have split to form four units.

SUMMARY

Alpine plants of *Achillea lanulosa alpicola* (Rydb.) Keck and *Potentilla diversifolia* Lehm. were transplanted to a lower altitude in Gunnison County, Colorado, and treated with a 0.01 per cent aqueous solution of gibberellic acid. Measurements of total height show increases of 120 per cent in *Potentilla* and 89 per cent in *Achillea* in the treated plants as compared to the controls. Leaf lengths in plants of both species are longer after applications of gibberellic acid, this increase being mainly the result of greater petiole length in the treated leaves as compared to the controls.

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